PhD Thesis:

A Novel Role for microRNA-214 in Inflammatory Smooth Muscle Cell Differentiation from Adventitia Stem/progenitor Cells and Arterial Remodelling

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Statement of originality

I, Shiping He, confirms that all research included in this thesis is my own experiment. The work carried out by collaborators and/or with the support of other colleagues has been properly indicated and acknowledged below along with my contributions. Previously published research papers are also acknowledged below.

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Abstract

Background: Cardiovascular disease (CVD) is still the leading cause of death in the developed as well as developing country. Atherosclerosis and neointima formation considered as chronic inflammatory diseases, in which many cells including inflammatory smooth muscle cell (iSMC) were involved. ISMC differentiation from vascular adventitia stem/progenitor cells (AdSPCs) has been recently recognised as a critical determinant in cardiovascular diseases. One study have recently reported an important role for microRNA-214 (miR-214) in mature vascular SMC functions and injury-induced neointima formation. However, little is known about the functional involvements of miR-214 in iSMC differentiation from AdSPCs and its contribution to adverse arterial remodelling.

Purpose: In this PhD study, I aimed to study the functional importance of miR-214 and its target gene in iSMC differentiation from AdSPCs and neointima SMC hyperplasia.

Results: miR-214 expression was significantly increased during SMC differentiation from AdSPCs in response to TGFβ1. miR-214 gain/loss-of-function assays showed that miR-214 plays an important role in SMC differentiation from AdSPCs. By co-incubating AdSPCs with TGFβ1 and TNFα, AdSPCs were induced to differentiate toward iSMCs as evident by a decreased expression level of SMC-specific genes, but an increased level of inflammatory genes in iSMCs. Importantly, such phenotype could be reverted by miR-214 over-expression. I observed that sonic hedgehog-glioma-associated oncogene 1 (Shh-GLI1) signal was closely modulated during iSMC differentiation, and demonstrated that Suppressor of Fused (SuFu) is the functional target of miR-214 controlling iSMC generation from AdSPCs. Further mechanistic studies revealed that increased amount of the GLI1 protein was translocalized into nuclei in miR-214 over-expressing or

SuFu knockdown cells, and the consensus sequence (GACCACCCA) of GLI1 within gene promoters of smooth muscle alpha-actin (SMαA) and serum response factor (SRF) was required for their regulation by miR-214 and SuFu. Additionally, Sufu also functions as a positive regulator for inflammatory gene regulations in iSMCs. *In vivo*, I demonstrated that locally enforced expression of miR-214 in the injured vessels significantly reduced SuFu expression level, iSMC generation, and inhibited neointima SMC hyperplasia after injury. Importantly, I observed that perivascularly transplanted AdSPCs significant contribute to neointima SMC hyperplasia by differentiating to iSMCs, and miR-214 over-expression could reverse such an effect. Finally, a decreased expression level of miR-214, but increased expression level of Sufu was observed in diseased human arteries. **Conclusions/Implications:** This thesis reports an unexplored role for miR-214 in iSMC differentiation from AdSPCs and controlling neointima iSMC hyperplasia. These findings provide new insights into the therapeutic effect of miR-214 in vascular diseases.

Key words: Adventitia stem cells; Progenitor cells, Arterial remodelling; Neointima formation; Atherosclerosis; Inflammatory smooth muscle cell; Smooth muscle cell differentiation, miR-214; microRNAs; Suppressor of Fused (SuFu).

List of Abbreviations

AAVs	Andeon-associate viruses
ABCA1	ATP Binding Cassette Transporter A1
AD	Alzheimer's disease
AGO/AGO3	Argonaute/Argonaute 3
AMI	Acute myocardial infarction
APS	Ammonium persulfate
Apo	Apolipoprotein
ATC	Anaplastic carcinomas
BACE1	beta-site amyloid precursor protein cleaving enzyme 1
BM derived	Bone Marrow derived
Bcl2	B-cell lymphoma 2
β-ME	β- Mercaptoethanol
CAD	Coronary Artery Disease
CAM	Cell adhesion molecule
CABG	Coronary Artery Bypass Grafting
CCL5	Chemokine (C-C motif) ligand 5
CD133	Prominin-1
c-kit	Mast/stem cell growth factor receptor/ SCFR
CHD	Coronary Heart Disease
ChIP	Chromatin Immunoprecipitation
CLL	Chronic lymphocytic leukemia
CTCL	Cutaneous T-cell Lymphoma
CO_2	Carbon dioxide
CXCL1	Chemokine (C-X-C motif) ligand 1
CVD	Cardiovascular Disease
DAPI	4'6-Diamidino-2-phenylindole

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DNM3	Dynamin-3 gene
EC	Endothelial Cells
ESC	Embryonic stem cell
ECM	Extra Cellular Matrix
EPC	Endothelial progenitor cell
EU	European
E-selectin	CD62 antigen-like family member E
FBS	Fetal Bovine Serum
Flk-1	Fetal Liver Kinase-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiform
GFP/eGFP	Green Fluorescent Protein/ Enhanced green fluorescent
HBSS	Hanks' balanced salt solution
HCV	Hepatitis C virus
HDL	High Density Lipoproteins
H_2O_2	Hydrogen peroxide
HF	Heart Failure
H/hr/hrs	Hour/hours
HEK293	Human Embryonic Kidney 293 cells
HLA-DR	MHC class II cell surface receptor
HIV	Human immunodeficiency virus
ICAM-1	Intercellular cell adhesion molecule-1
IF	Ischemia Failure

iSMC	Inflammatory Smooth muscle cell
IFN	Interferons
IL	Interleukins
IR	Ischemia reperfusion injury
ISO	isoproterenol
JAM-A	Junctional adhesion molecule-A
KLF2/4	Kruppel-like factor 2 and 4
LDL	Low Density Lipoprotein
Ly6C	Lymphocyte antigen 6 complex
LVR	Left ventricular remodeling
Ox-LDL	Oxidized LDL
MAC-1	Monocyte adhesion receptor-1
miR/ miRNA	MicroRNA
mRNA	Messenger RNA
miR-214	MicroRNA-214
MCP-1	Monocyte chemoattractant protein 1/CCL2
Mac-1	Integrin alpha-M/CD11
Min	Minute
MI	Myocardial Infarction
MSCs	Mesenchymal stromal cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	Non-communicable diseases
NF-ƙB	Nuclear factor kappa-light-chain-enhancer of activated B
NO	Nitric Oxide
NDDs	Neurodegenerative disorders
PBS	Phosphate buffer saline
P body/ PBs	Processing bodies

PCI	Percutaneous Coronary Interventions
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PE	Phenylephrine
piRNAs	Piwi- associated small RNAs
PLA	Phospholipase A
Pol II	RNA polymerase II
Pre-miRNA	Precursor micro RNA
Pri-miRNA	Primary micro RNA
PTC	Papillary carcinomas
PTCA	Percutaneous Transluminal Coronary Angioplasty
PTEN	Phosphates and tensin homolog
P-selectin	Platelet Activation-Dependent Granule to External Membrane Protein
P/S	Penicillin- streptomycin combination
RNA	Ribose nucleic acid
RNAi	RNA interference
RNase III	Ribonuclease III
RISC	RNA Induced Silencing Complex
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
q-PCR	Real time Polymerase Chain Reaction
Sca-1^{+}	Stem Cell Antigen 1 positive cells
SCC	Squamous cell carcinoma
Sec	Seconds
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean

shRNA	Short hairpin RNA
siRNAs	small interfering RNAs
SM22α	Smooth Muscle 22 α
SmαA	Smooth Muscle alpha Actin
SMC	Smooth Muscle Cells
SM-MHC	Smooth Muscle Myosine Heavy Chain
SREBP	Strol regulatory element-binding protein
SRF	Serum Response factor
SSEA-1	Stage-specific embryonic antigen-1
STAT	Signal Transducer and Activator of Transcription
Sufu	Suppressor of fused homolog
TAC	Transverse aortic constriction
TGFβ1	Transforming Growth Factor β
TNFα	Tumour necrosis factor α
TRBP	TAR RNA binding protein
UTR	Un-translated region
VLA-4	Integrin α4β1/ Very Late Antigen-4
VSMC	Vascular Smooth Muscle Cells
VCAM-1	Vascular cell adhesion molecule-1
VWF	Von Willebarnd factor
WHO	World Health Organization
XBP1	X-box binding protein1
αMHC	α-myosin heavy chain

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1. Introduction

Although huge improvement on treating and researching into cardiovascular disease (CVD) has been successfully achieved in the past several decades, CVD is still the leading cause of death around the world (Benjamin et al. 2017). A recent report on CVD in Europe, of all the deaths registered in 15 EU countries, one third of deaths were caused by CVDs (Townsend et al. 2016). According to statistical analyses by the World Health Organization (WHO), more than 17.3 million people died from CVDs in 2013 all around the world, a 41% increase since 1990 (Roth et al. 2015). A rapid increase in world population growth and number of aging people, are considered the two major contributing factors to this increase. Additionally, the composition of CVD patients in recent years has changed over time. Increasingly more and more young patients present with signs of severe CVD onset. In 2002, The WHO reported that more than 46% of deaths due to CVD were in patients under 70 (Guilbert 2003). These global statistical analyses reveal the serious threat that CVD presents to human's future.

Raised blood pressure, tobacco, excess alcohol consumption, cholesterol and being overweight are the major risk factors of CVD. For instance, tobacco smoking results in endothelial cell dysfunction, which plays a critical role during the atherosclerosis process, a precursor to CVD onset. Evidence from clinical and pre-clinical studies supports that smoking tobacco promotes endothelial cell dysfunction which not only initiates atherosclerosis but also accelerates it (American Heart Association Statistics 2004). Atherosclerotic plaque formation and/or growth will lead to the blood vessel narrowing or even complete occlusion, resulting oxygen depletion and cell death in tissues and organs further downstream.

Atherosclerosis is the main pathophysiological process of most ischemic CVDs. According to investigations in 2013, ischemic heart disease is the main cause of increased death in CVDs (Roth et al. 2015).

Atherosclerosis is considered as a chronic inflammatory disease, with a series of pathological changes and rampant cell proliferation occurring at the lesion site resulting in a narrowed artery or complete blockage, leading to diminished blood flow to the myocardium. Coronary artery disease is one of the most serious consequences of atherosclerosis. Surgical operations like angioplasty, Percutaneous transluminal coronary angioplasty (PTCA) with stenting, atherectomy and coronary artery bypass grafting (CABG) are widely used in the clinic to treat patients who are suffering from the consequences of severe, end-stage coronary atherosclerosis (Weintraub 2007). PTCA, first developed in 1977, is the most successful and minimally invasive surgery for treating patients with ischemic coronary artery disease (CAD). It has since become one of the most commonly used surgical techniques in the US, accounted for 3.6% of all surgical procedure in US in 2011(Weiss, et al. 2006). Although it was widely used in the clinic, PTCA is not flawless, as there are many side effects associated with this technique post-surgery. These unavoidable side effects include restenosis of the vessel wall. Novel research has focused on improving this procedure or even promoting the use of alternative non-invasive strategies, in order to help prevent current side effects from arising. For example, since PTCA requires the use of a balloon-tipped catheter, which is inserted into the blocked site, an anti-proliferation drug coated- balloon was developed to reduce the proliferating cells from blocking the site once more. One clinical trial used paclitaxel-coated balloons before treatment, and compared the therapy prognosis against the drug eluting stents treatment patients.

Promising data were found that this method has better outcome than using the foreign body replacement stent (Mohiaddin et al. 2018). Restenosis of the vessel wall or neointimal hyperplasia has been considered as the most prominent pathological issue with the patients after PTCA and stenting. In brief, restenosis is a maladaptive response to arterial injury and characterised by arterial neointimal formation. Vascular smooth muscle cells (VSMCs) play a critical role during this process, and it has been reported that VSMCs participate in restenosis and neointimal formation by undergoing phenotype switching, proliferation, migration as well as by secreting extracellular matrix (ECM) proteins into the injured intimal layer(ER et al. 2011). A clinical report shows that over 20-50% of patients after balloon angioplasty and about 10-30% who received a stent during the procedure experienced restenosis (Bhargava et al. 2003). The underlying pathophysiology of restenosis involves a very complex series of cellular and molecular mechanisms, which are not yet fully understood. Thus, the treatments for restenosis are far from satisfactory. Many cells involved into the atherosclerosis are also involved in neointimal formation following PTCA. Endothelial cells, macrophages, VSMCs and myo-fibroblasts are among the most important cell types to participate in neointimal formation. Research focusing on these cells not only reveal the exact roles that these cell play during the pathological process but also provide a novel therapeutic direction for the future clinical treatments (Gimbrone et al. 2016; Bennett, et al. 2016; Groh et al. 2018). VSMCs are the main cellular component of the vascular media. Under normal physiological conditions, VSMCs maintain the vascular structure and rarely undergo proliferation. However, under specific environmental stimulation, synthetic VSMCs are apparent and participate in various pathological processes. Inflammatory SMCs (iSMCs) identified in the developing restenotic lesion were found

resident in the intima of the vessel, producing collagen, elastin and various inflammatory cytokines. Altogether, this was shown to promote hyperplasia of the neointima, resulting in arterial wall thickening and vessel narrowing and/or occlusion. Understanding the source of iSMCs during restenosis is of great importance for future atherosclerosis and restenosis therapy.

Many types of the cells have been reported as capable of contributing to iSMC such as; mature VSMCs migrating into the intima and undergoing phenotype switching; endothelial cells (ECs) undergoing mesenchymal transition, circulating stem cells as well as the vascular stem/progenitor cells.

Adventitial stem/ progenitor cells (AdSPCs) are considered as one of the cellular sources of iSMCs in atherosclerosis and restenosis. In 1997, one group identified a group of $CD34^{+}$ endothelial progenitor cells (EPCs), in peripheral blood, which participate in new vessel formation (Asahara et al. 1997). Many adult stem progenitor cells have been reported to be involved in CVDs. Previously, the adventitia of the blood vessel was considered to consist mainly of fibroblasts, connective tissue matrix as well as nerve ending. In 2004, one group examined organs and tissues of ApoE deficient mice and found that many stem cells that were detectable through antibody selection were present in the adventitia of the vascular wall. These cells were positive for Stem cells antigen-1 (Sca-1), proto-oncogene c-kit, CD34 and Flk-1, but negative for the bone marrow biomarker CD133 and embryonic stem cell marker SSEA-1. Further experiments indicated that these cells could be differentiated into SMCs *in vitro* as well as *in vivo* (Hu et al. 2004). Interestingly, when these Sca-1 positive cells were transferred

to the adventitial side of the vein graft in ROSA26/ApoE$^{-/-}$ mice for 4 weeks, the cells could be traced to the neointimal of the blood vessel, which indicated that the Sca-1 positive progenitor cells were able to migrate from the adventitia to the neointima and more importantly they were able to differentiate into SMCs (Hu et al. 2004). Moreover, miRNAs were found to play a crucial role during the differentiation of Sca-1 positive stem progenitor cells into SMCs (Zhao et al. 2015), suggesting that certain miRNAs can influence adventitial stem progenitor cells differentiation, and thus have an important effect on several disease processes.

MiRNAs are a group of small non-coding RNAs, which are found in a variety organism including viruses, plants as well as metazoans. The main function of miRNAs is to act as post transcriptional regulators. Mature miRNA, along with the relative Argonaute family protein, is loaded onto an RNA-induced silencing complex, which then binds to a recognised 3'UTR region of its target mRNAs. MiRNAs are thus able to control protein expression within the cell by determining the fate of its target mRNA molecule which will eventually undergo cleavage, degradation, or transport and storage into P bodies thereby inhibiting protein translation, a fundamental biological process. Numerous miRNAs have been proven to take part into almost every stage of the atherosclerotic process and neointimal formation, including the regulation of endothelial dysfunction, cholesterol homeostasis, plaque development, neo-angiogenesis, VSMC migration and phenotype switching, as well as iSMC differentiation during the restenosis (Madrigal-Matute, et al. 2013). Future gene therapies and/or pharmacological compounds should take advantage of this group of non-coding RNAs to improve the outcome for patients with CVD.

1.1 Cardiovascular Diseases (CVDs)

According to statistical analyses by the WHO global status report on non-communicable diseases (NCDs), CVD was the leading cause of NCD death in 2012, accounting for 17.5 million deaths (Mendis, et al. 2015). It remain one of the most serious diseases in Europe accounting for about 4 million deaths in 2016, which is around 45% of total mortality in the EU (Townsend, et al. 2016), Unfortunately, this phenomenon is now spreading to low and middle income countries as well (Lopez, et al. 2006). According to data from 2016, regions affected by the highest rates of CVD were recorded in the central Asia and Eastern Europe (Thomas, et al. 2018), with nearly 70% of deaths attributed to CVDs occurring in low and middle income countries(Figure 1) (Benziger, et al. 2016).

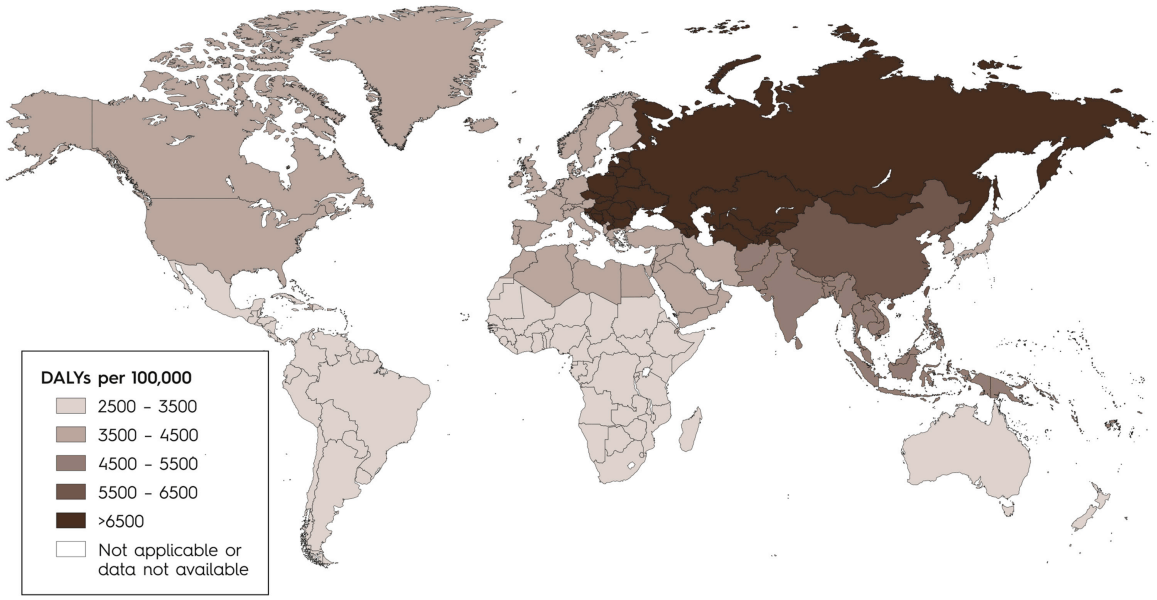


Figure 1. Eastern Europe and Central Asia region contribute to the highest per capita CVDs burden. According to the data of change in CVDs disability adjusted life years (DALYs) in every 100,000 people in 21 global region investigation between 2000 and 2016 (Benziger, Roth, and Moran 2016) (Thomas, et al. 2018).

Risk factors attributable to besides gender, age, ethnicity, family history and genetic makeup, include; high systolic blood pressure, diet, high total cholesterol, air pollution, tobacco, high body mass index, high fasting

plasma glucose, impaired kidney function, low physical activity, alcohol and drug use, and other environmental and occupational risks.

Diet, high blood pressure and tobacco are the top three leading risk factors for CVDs (Figure 2), whilst the remaining CVD risk factors vary from region to region. For example air pollution presents a much greater risk in East Asian and South Asian countries while alcohol consumption appears to be a more critical risk factor in Eastern Europe(Benziger, et al. 2016).

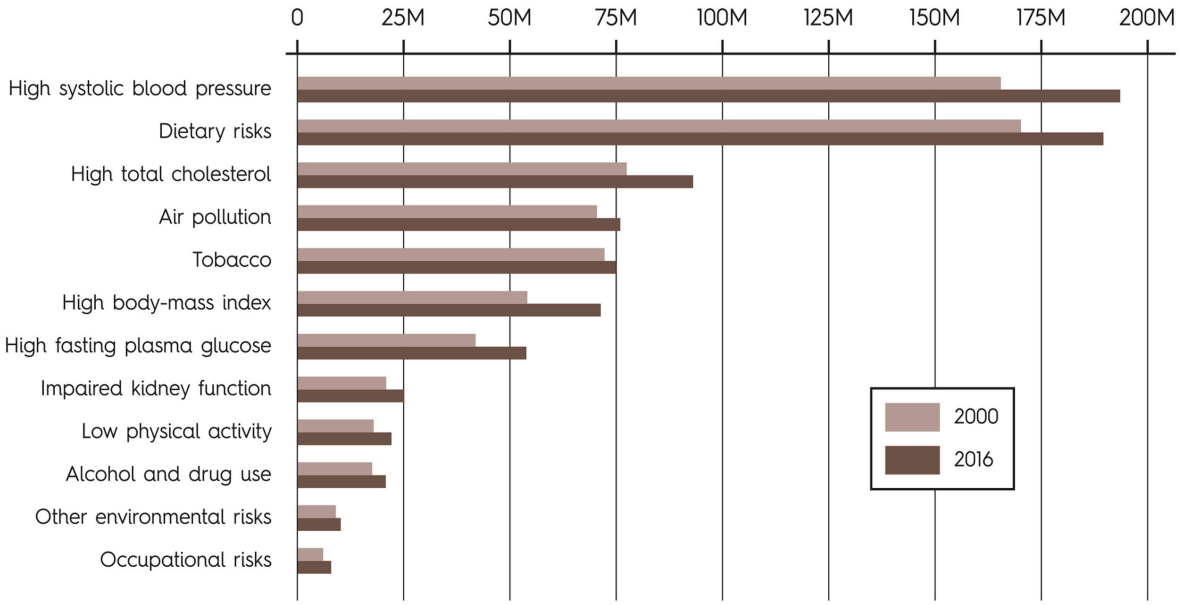


Figure 2. CVDs risk factors contribute to the number of DALYs, between 2000 and 2016 including both sexes, all around the world (Thomas, et al. 2018).

The cardiovascular system is one of the most complex systems in the human body, and many diseases are linked to CVD. In the clinic, a number of diseases are classed as CVDs including; coronary heart disease, heart failure, cardiac arrest, ventricular arrhythmias, sudden cardiac death, rheumatic heart disease and transient ischemic attack, subarachnoid ischemic stroke, intracerebral haemorrhage, abdominal aortic aneurysm, peripheral artery disease and congenital heart disease (Wong, et al. 2014). Ischemic heart diseases, belonging to the coronary heart diseases (CHD) category,

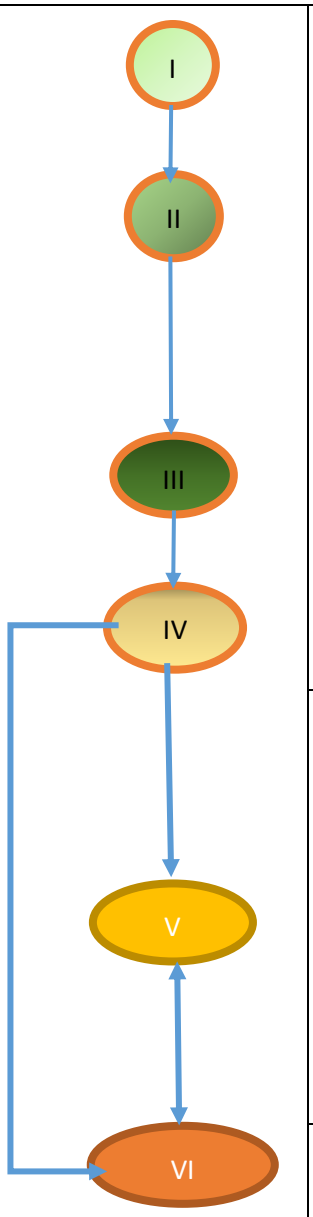
contributed to approximately 46% of CVD deaths in males and 38% of deaths in females in 2011(Shanthi Mendis. 2011). In 2016, the number of people suffering from ischemic heart disease was estimated to be about 49% of CVD cases (Thomas, et al. 2018). CHD occurs when the coronary arteries carrying oxygenated blood and nutrients to the heart (or myocardium) become blocked or narrowed by an atheroma, a process caused by the clinically defined process of 'atherosclerosis'.

1.2 Atherosclerosis and restenosis

Atherosclerosis is the leading cause of ischemic CVDs. It is recognized as a chronic inflammatory disease within the arterial wall. Atherosclerosis lesions normally develop in large and medium size arteries which can lead to ischemia of the heart, brain as well as extremities of the body (Ross. 1999). The exact triggers for atherosclerosis are still unknown, but similar to all the CVDs, tobacco smoking, dietary risks, high blood pressure, obesity and high total cholesterol including high or low-density lipoprotein cholesterol and low level of high-density lipoprotein in the blood system are known to be associated with an increased risk of atherosclerosis. Health behaviour plays an important role in delaying or preventing atherosclerosis. For example, a group tracked about 9776 adults in US for an average of 19 years, and they concluded that a higher intake of dietary fibre could reduce the risk of CHD in most cases (Bazzano, et al. 2003). Based on the histological analysis, the stages of atherosclerotic lesion formation can be classified into six different morphological types: 1) initial lesion which predominantly consists of macrophages and scattered foam cells surrounding the atherogenic lipoprotein (Stary, et al. 1992). 2) progression-prone type II lesion; with the macrophage foam cells and lipid laden smooth muscle cell (SMC) as the key feature, and a fatty streak as the primary lesion

structure. 3) Intermediate lesion, this is the stage between type II and type III, which is similar to type II but contains scattered extracellular lipid drops and the SMC coherence were blocked by some particles (Stary, et al. 1994). 4) Extracellular lipids are extensively distributed throughout the atheroma in the region of the intima known as the lipid core. 5) Type V lesion (including V a, V b and V c type), contains new, fibrous connective tissue, with the presence of a fibro-atheroma known as type Va, the presence of lipid core calcification known as type Vb, or minimal presence (or even absence) of a lipid core referred to as type Vc. 6) Type VI lesion is the final morphology of the atherosclerosis, which usually results in disruption of the lesion surface, hematoma, haemorrhage and the thrombotic deposits. Notably, the type IV or V morphology superimposed one or more character described above will recognized as the type VI lesion (Stary, et al. 1995). (Table 1)

Table 1. Different types of atherosclerotic lesion and its relationship

Nomenclature	Histological character classification		Sequences in progression	
Type I lesion	Initial lesion			Growth maily by lipid accumlation
Type II lesion	Progression –prone type II lesion (type II a lesion)	Fatty steak		
	Progression – resistant type II lesion (type II b lesion)			
Type III lesion	Intermediate lesion			
Type IV lesion	Atheromatous plaque			
Type V lesion	Fibroatheroma (Type V a lesion)	Fibrolipid/fibrous plaque		Mainly display SMCs and collagen increase
	Calcific lesion (Type V b/VII lesion)	Calcificed plaque		
	Fibrotic lesion (Type V c/VIII lesion)	Fibrous plaque		
Type VI lesion	Lesion with surface defect with/without hematoma haemorrhage, thrombotic deposit			Thrombosis, Hematoma

This table displays the pathways in the progression of human atherosclerotic lesion. The arrows show the sequence of morphologies changes over time. With the main change pertaining to lipid accumulation from type I to IV. The transition between stage IV, V and VI depends on lesion thickness increase as thrombotic materials accumulate on their surface (Stary, et al. 1995).

Surgery operations like angioplasty, PTCA with stenting, atherectomy and coronary artery bypass grafting (CABG) are widely used in the clinic to treat patients who suffer from severe coronary atherosclerosis (Weintraub. 2007). Among these surgical operations, CABG and PTCA are the most popular approaches in clinical treatment for revascularization. PTCA, also known as percutaneous coronary intervention (PCI), was first developed in 1977 and became one of the most common surgical techniques in the US (Weiss, et al. 2006). In Canada, the popularity of the revascularization operation has increased continually to about 50% of 40,000 CHD patients in each year (ER, et al. 2011). Although it is widely used in the clinic, PTCA is not a flawless surgery and side effects like the restenosis of the vessel wall are inevitable.

Although atherosclerosis and restenosis are defined as different clinical diseases, they still involve similar pathological processes such as neointima formation. Neointimal formation is a common pathological process in different cardiovascular diseases such as atherosclerosis, post angioplasty restenosis, coronary heart diseases and so on (Ji, et al. 2007). Atherosclerosis, neointimal formation and hyperplasia as well as the restenosis are continual problems, which hinder clinical therapy. No matter what stage of atherosclerosis is, the artery injury caused by surgery or disease itself induced the maladaptive response of the artery are likely develop into the restenosis. Restenosis is defined by the diameter of the stenosis. When the vessel diameter reaches around 50% reduction after the treatment this is termed as vascular restenosis (Bhargava, et al. 2003). Usually, the lumen loss after the revascularization can be divided into three different stages; such as early loss of relative elastic recoil, late loss of relative elastic recoil causing negative remodelling and neointimal

hyperplasia(Weintraub. 2007). It has been reported that about 20-50% of balloon angioplasty surgery patients and 10-30% stent implantation patients are likely to develop restenosis. Restenosis usually presents between one and three months following balloon angioplasty surgery, with angina occurring in the fourth month, but this is delayed by one to three months in patients who have received stents (Serruys, et al. 1988). Although endovascular stents are considered to delay restenosis in the early stages after the operation, implantation of a foreign object has the added risk of inducing an inflammatory response, which plays an important role during neointimal hyperplasia. Clinical research found that patients receiving stents, develop a thicker neointimal layer and display greater inflammatory cell infiltration (Farb, et al. 2002). Some scientists consider Restenosis as the Achilles' heel of coronary angioplasty, which may ultimately lead to failure of the surgical operation. The origin and identity of cells types that contribute to restenosis is not well understood. SMCs are the most dominant cell types within the neointima. Whilst the traditional view is that these cells come from the medial layer of the vessel through vascular migration, more recent evidence suggests that the origin of the SMCs in restenotic lesions is much more complex than we previously thought. Bone marrow progenitor cells and adventitial stem progenitor cells are regarded as key additional contributors to SMCs during the restenosis besides the medial infiltration of SMCs (Bentzon, et al. 2006) (Tigges, et al. 2013).

1.3 Risk Factors for the atherosclerosis and restenosis

Risks factors considered to promote atherosclerosis and restenosis, include an unhealthy diet, physical inactivity, tobacco use and excessive alcohol

consumption which can lead to a set of clinical symptoms such as hypertension, hyperlipidaemia, diabetes, obesity and hemodynamic forces (Organization. 2017). Atherosclerosis leads to the development of atheromatous plaques in the inner layer of the arteries. Among all these risk factors, lipoproteins especially oxLDL levels have been recognized to play a central role during the pathogenesis of the atherosclerosis plaques. In the circulatory system, cholesterol is transported and removed from cells and tissues by lipoprotein. Excess lipoprotein trapped in the area where the blood flow is disturbed may trigger an inflammatory response and recruit monocytes, activate underlying ECs and VSMCs, which triggers the process of the atherosclerosis (Tabas, et al. 2015). This earliest lesion of atherosclerosis are called fatty streaks and commonly appear in children or during infancy (Pant, et al. 2014). In this stage, macrophages and SMCs are recruited to the site where lipids are trapped and attempt to scavenge the excess lipids. As the atherosclerosis develops, the fatty streak becomes an advanced atherosclerotic lesion, consists of macrophages/foam cells, SMCs and inflammatory cells. Lipid uptake will induce foam cell death and attract more macrophages to the plaque and further promote atherosclerosis development. As described in Table 1, the early stages of the atherosclerosis are mainly characterised by lipid accumulation. So obviously that dyslipidaemia is a critical risk factor for atherosclerosis development (Laffont and Rayner. 2017).

Reactive oxygen species (ROS) are considered another important initiator of the atherosclerosis. The human body is oxygen-centric and oxidative phosphorylation (as well as non-respiratory oxygenation) occurs in every cell every minute (Miller, et al. 2011). ROS is a by- product of the oxidation reaction and enzymes such as NADPH oxidases generate ROS during normal

aerobic metabolism in cells (Droge. 2002). As mentioned previously, smoking is one of the top risk factors for CVD. It has been reported that cigarette smoke releases a large number of free radicals into the circulatory system, which activates dysfunctional nitric oxide (NO) biosynthesis (Barua, et al. 2003). Excess free ROS can promote oxidation of LDL and promote the LDL induced EC and VSMC inflammation within arteries. Furthermore, it has been shown that ROS induces a set of inflammatory signally pathways; for instance, IL-4 induced MCP-1 expression in a ROS-dependent manner in ECs. Increased MCP-1, allows ECs to recruit a large number of monocytes into the site of the vessel wall and promotes migration of monocytes into the sub-endothelial region(Lee, et al. 2010).

The restenosis depends on variety factors, including the patient's additional health complications, lesion characteristics and procedural variables. The patient's condition could affect restenosis development if for instance they present with related diseases (such as diabetes mellitus (DM)) or have undergone surgery for restenosis at another treated site, or have previously required intervention for acute coronary syndromes. DM can increase the likelihood of restenosis in patients, as metabolic alterations resulting from DM could trigger endothelial dysfunction, strengthen the production of growth factors, increase the likelihood of platelet aggregation and promote thrombogenicity(Aronson, et al. 1996). A clinical study showed that in a cohort of 954 patients who received PCI surgery, over 28% of insulin-dependent diabetes patients needed revascularization compared with 16.3% non-diabetic patients (Abizaid, et al. 1998). For acute coronary syndrome, injury to vessel wall will promote cellular proliferation and worsen the existing microenvironment. Lesion characteristics and procedural variables including the diameter of the vessel, the length of the lesion or stent,

minimum lumen diameter before and after the stenting, the ostial lesion, saphenous venous graft and total occlusion are critical in determining the lesion constitutions (Bhargava, et al. 2003). Similarly, the most important predictor of restenosis is the size of vessel diameter and the length of the lesion. It has been reported that a larger minimum lumen diameter after stent implantation decreases the risk of restenosis in patients (Moussa, et al. 1999). Additionally, some genetic factors such as the polymorphisms in genes coding for angiotensin converting enzyme, glycoprotein receptor IIIa PLA1/2 and haptoglobin2/2 have been reported associated with a higher risk of restenosis (Ribichini, et al. 1998; Di Castelnuovo, et al. 2001; Roguin et al. 2001).

1.4 The pathology of atherosclerosis and restenosis

Most blood vessel is composed of three distinct layers of cells, called tunica intima, tunica media and tunica adventitia (Gutterman. 1999). The tunica intima includes a single layer of vascular ECs resting on a basement membrane, which is mainly composed of internal elastic lamina membrane along with some collagen type IV and laminin proteins. The medial layer is mainly composed of VSMCs embedded in the ECM. The adventitial layer is the outside layer of the vessel, and contains connective tissue, collagen fibres and fibroblasts, a vasa vasorum, fat cells as well as some inactive inflammatory cells (Gutterman. 1999). Interestingly, up until recently the primary function of adventitial layer was considered to protect and provide nutrition for the medial and intimal layers. However, the region of the adventitia facing the SMC layer, known as the 'vasculogenic zone', contain a number of endothelial progenitor cells, as well as some multipotent stem and progenitor cells, and contributes to new blood vessel formation as well

as providing a source of inflammatory cells for a local immune response (Zengin, et al. 2006). These cells, as well as mesenchymal cells, work in concert to maintain blood vessel wall homeostasis, repair the damaged vessel as well as respond to inflammatory stimulation.

As previously discussed, atheroma formation occurs as a result of physical and/or chemical abnormalities induced by hypertension, hyperlipidemia, hyperglycemia, elevated ROS levels, and an activated endothelial which triggers an inflammatory cascade in the sub endothelial layer (Kojda et al. 1999). Meanwhile accumulated lipoprotein also triggers the ECs to release inflammatory chemokines, which help to recruit a set of highly specific cells such as monocytes and macrophages into the site. In an attempt to engulf the accumulated cholesterol, macrophages become inflammatory foam cells and recruit more cells into the growing atheroma (Laffont et al. 2017). Besides, the oxidized low-density lipoproteins also promote the activated ECs to upregulate adhesion molecule expression which boost the inflammatory cell accumulation (Butini et al. 1994). Additionally, endothelial dysfunction also activates platelets which accumulate at the pathological site, releasing platelet derived growth factor (PDGF) and inducing SMC migration from the medial layer into sub-endothelial space, which then start to undergo proliferation and phenotype switching(Libby 2002) (Mack. 2011). Once enough cells have accumulated, the lesion progress into a mature atheroma with the core of the atheroma consisting of foam cells and extracellular lipid droplets. The SMCs together with some collagen-rich matrix form a cap surrounding the lipid core and other cells including macrophages, T cells and mast cells located in the shoulder region of the atheroma. This structure is not stable, and as the atherosclerosis

progresses, increased cell apoptosis and iSMCs accumulation results in the calcification of the VSMCs which further promotes atherosclerotic plaque growth. Also if the lipid and the inflammatory molecules keep clustering to the site, macrophages will replace the SMCs in the cap of the atherosclerosis plaque which will increase the likelihood of plaque rupture (Yu, et al. 2018). Angiogenesis and haemorrhage within the core of the lesion could drive the vulnerable plaque to rupture which may lead to further cardiovascular complications such as stroke and heart attack (Madrigal-Matute, et al. 2013).

Neointimal formation and hyperplasia are complicated cellular and molecular responses which occur in many CVDs like atherosclerosis and hypertension, as well as after vascular surgery such as the PCI and CABG. This kind of disorder induces the arterial wall to thicken and thus cause further reduction of the vessel lumen size. It is widely reported that SMCs undergo migration, phenotype switching and abnormal proliferation in the intimal region, which plays a fundamental role in development of the neointima and restenosis. Other cell types such as myofibroblasts, ECs and macrophages are also involved in this process. Although numerous studies have been carried out on the pathogenesis of the neointima formation, the underlying mechanisms of neointima SMC hyperplasia are still not completely understood. Histological studies found that in the restenosis site, the inflammatory cells followed by the accumulate of the synthetic SMCs or iSMCs were exist, the myofibroblast and the iSMCs secret huge amount of extra cellular matrix (ER, et al. 2011). The initiation of the neointima formation and hypertension during the restenosis is said to start from the maladaptive response to arterial trauma caused during the

angioplasty, consisting of thrombosis, inflammation, cells migration and proliferation as well as the extra cellular matrix secretion, within 6 months of surgery (Nikol, et al. 1996). In short, about three distinct stages are used to classify the loss of luminal diameter after angioplasty, that is early loss elastic recoil of the vessel, negative remodelling, and neoinitimal formation and hypertension (Weintraub. 2007). Compared with the atherosclerosis process, neointimal formation in restenosis is much simpler and more efficient. Whilst atherosclerosis is initiated from a very young age and may develop into a serious CVD after many years, restenosis can occur within the space of 6 months. Elastic recoil is a dynamic phenomenon that starts immediately after the angioplasty operation and is characterised by instant luminal diameter loss. It was found upon clinical observation that in about 34% of cases loss the lumen diameter occurred within 15min of balloon inflation(Caixeta, et al. 1996). Stent implantation may have a more reduced rate of elastic recoil, but a suboptimal stent insertion has been found to cause average of 60% less of the desired lumen dimension (Bermejo, et al. 1998). In total, immediate elastic recoil causes about 50% of the restenosis during the angioplasty (Rozenman, et al. 1993). Platelet adhesion, activation and thrombosis are the key step in the next stage of the neointimal formation. This step also starts immediately after the angioplasty, with the fracture of the atherosclerotic plaque and direct exposure to blood flow, leading the thrombogenic contents in the plaque interacting with platelets and thereby inducing the platelet adhesion and as a result, thrombosis (Casscells, et al. 1994). Furthermore, dysfunction of the endothelial cells result in less express some antithrombotic factors such as NO and prostacyclin in the fractured site which promotes further platelet adhesion and aggregation after surgery (Weintraub. 2007). After that, activated platelets release plenty of mitogens like the PDGF, serotonin and

thromboxane A2 that will induce SMC proliferation and migration. Many different cell types contribute to iSMC accumulation in the neointima including the medial SMCs, ECs, circulating stem progenitor cells and most importantly, adventitial stem progenitor cells. Vascular remodelling exists in both atherosclerosis and restenosis, but the characterisation of these two pathological processes differ significantly. During atherosclerotic plaque formation, outward vascular remodelling, namely external elastic membrane hyperplasia, will be triggering to maintain the structure of the lumen (Glagov, et al. 1987). In contrast, neointimal formation, hyperplasia and concentric compression in the intima layer of the vessel are the main processes by which restenosis occurs. As discussed previously, iSMC accumulation into the intimal layer and narrowing the lumen of the vessel occurs at the same time as adventitial thickening and concentric compression possibly due to collagen secretion which replaces the original hyaluronic proteins (Mintz, et al. 1996). Notably, although the endovascular stent could reduce early elastic recoil and lumen loss in the early stage of the restenosis, investigations found that stent implantation induce inflammatory cell infiltration and triggers an inflammatory response which promotes neointimal hyperplasia (Farb, et al. 2002).

1.5 Cells involved in atherosclerosis and restenosis

As previously mentioned, atherosclerosis can start at a very young age but only begins to affect patients' quality of life after many years. Restenosis usually occurs after a surgical operation, of which the main characteristic is neointimal hyperplasia. Numerous cells are involved in this process, especially ECs, VSMCs, macrophages as well as some stem and progenitor cells.

1.5.1 Endothelial cells

In brief, the endothelium is a cellular monolayer which acts as a perfect functional barrier for the blood and surrounding tissue by regulating blood fluidity, maintaining vessel wall integrity, controlling the vascular tone and regulating permeability of the vessel wall(Pober, et al. 2007). ECs also involved in vasoactive mediator synthesis and degradation, lipoproteins transport and metabolism, ECM secretion, growth factors and cytokines biosynthesis as well as the vascular inflammatory response(Gimbrone, et al. 2016). Under pathological conditions, pro- inflammatory factors activate ECs which induce several gene activation pathways and adjust many normal functions in response to potentially harmful stimuli.

Endothelial dysfunction is the initial trigger for the atherosclerosis and restenosis. Moreover, it has been proven that many pathophysiological processes could induce and enhance endothelial dysfunction including hypercholesterolemia, diabetes, hypertension, oxidative stress, ageing and pro-inflammatory cytokines (Gimbrone, et al. 2016). As previously mentioned, ROS and lipoproteins activate ECs, initiate the inflammatory cascade and promote pro-inflammatory cytokine expression. ECs trigger atherosclerosis and neointima formation through multiple cross-talking signalling which amplify the effect of endothelial dysfunction in atherosclerosis process. For example, EC damage or dysfunction will recruit the leukocytes and monocytes to the lesion site, meanwhile active ECs upregulate cells adhesion molecule expression on the surface of the cells to anchor the immune cells. The most important adhesion molecule is intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion

molecule-1 (VCAM-1). VCAM-1 was recognized as a selective adhesion molecule and has highly selective adhesion ability on monocytes and lymphocytes by interact with the VLA-4 receptor (Elices, et al. 1990). It has been proven that hydrogen peroxide treatment could upregulate the ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells (HuVECs) within 6 hours, and neutralizing ROS during the treatment could reverse this elevation (Habas, et al. 2018). Another group also demonstrated that treatment of rabbit aortic endothelial cells with oxidized lipoproteins, like lysophosphatidic choline, induced ICAM-1 and VCAM-1 expression and further increased the adhesion of blood monocytes (Kume, et al. 1992). *In vivo* experiments also demonstrated that reduced VCAM-1 and ICAM-1 gene expression in ApoE-null mice could reduce aortic lesion area in a murine model of atherosclerosis (Cybulsky, et al. 2001; Bourdillon et al. 2000).

Endothelial activation also induces secretion of a set of chemokines such as IL-1, MCP-1, IL-8 and E-selectin (Libby, et al. 1986; Cushing, et al. 1990; Pober et al. 2007). Furthermore, EC activation causes a rise in intracellular Ca2+, upregulating P-selectin expression, a molecule is functionally similar to E-selectin, which in turn promote neutrophil trans-endothelium migration (Lorant, et al. 1993; Pober, et al. 2007). These intercellular autocrine and paracrine signalling events in the activated ECs could activate neighbouring ECs and recruit more circulating leukocytes into lesion site, triggering a more complex immune response network in the early period of the atherosclerosis(Pober, et al. 2007).

It is worth noting that the NF-ƙB signalling pathway seems play a central role in the endothelial pro-inflammatory activation process. NF-ƙB regulates many target genes involved in atherosclerosis, including adhesion molecules, cytokines and chemokines. NF-ƙB also promotes cell proliferation and cell survival-related gene expression. Interestingly, NF-ƙB also upregulates expression of the antagonistic gene, IkBa, to limit or abolish NF-ƙB cascade and ensure that active cells return to their quiescent state. Many cytokines and chemokines which are expressed by active ECs as previously mentioned, such as ICAM-1, VCAM-1, MCP-1, IL-8 (Linghu, et al. 2019) as well as the E-selectin, which is produced by type II activation of ECs (Pober, et al. 2007), are regulated by NF-ƙB signalling. VCAM-1 is a selective adhesion molecule that is expressed a very low level in quiescent ECs. Gene structure analysis found that the promoter of the VCAM-1 gene contains two NF-ƙB binding sites which are critical for VCAM-1 expression (Collins, et al. 2001). Another experiment also shows that eucalyptol, a monoterpene known as an anti-inflammatory component, could reverse the acute inflammatory reaction in HuVECs and reduce the VCAM-1 expression by blocking the degradation of the NF-ƙB antagonistic gene, IkBa (Linghu, et al. 2019). Oxidative stress and dyslipidaemia are two major triggers of NF-ƙB activation in endothelial dysfunction. Other factors such as hypertension and homocysteine also play a very important role in NF-ƙB activation in atherosclerosis (Collins et al. 2001). Multiple transcription factors such as Krüppel-like factor 2 and 4 (KLF2/4) have also been reported to be involved in endothelial dysfunction, and exert an anti-inflammatory effect in atherosclerosis (Atkins, et al. 2008; Zhou, et al. 2012).

Endothelial dysfunction also acts as a candidate for triggering neointimal formation during the restenosis. In the normal circulating system, the endothelium not only acts as an interface between blood and the vessel wall, it also participates in regulating vascular wall homeostasis. Endothelium provides an antithrombotic surface which separates blood flow from matrix proteins in the sub endothelial space and interacts with SMCs in the medial layer to regulate cells proliferation, migration and death. Endothelium can release mediators like NO and prostacyclins, which can inhibit platelet aggregation and adhesion to the intimal surface under normal physiological condition (Tesfamariam. 2016). However, these mediators are dysregulated upon endothelial activation, causing platelet adhesion/aggregation, PDGF production, adhesion molecule upregulation, monocyte/macrophage recruitment and activation, which in turn initiate pro-inflammatory signal cascades and eventually promotes neointimal lesaion. The activated endothelium also affects the intimal ECM mass by controlling their turn-over through secreting proteases and relevant inhibitors.

Intimal surface injury or endovascular device implantation can trigger platelet aggregation and activate an inflammatory response. Injured ECs recruit neutrophils and monocytes into the subendothelial space and release cytokines such as IL-1β, IL-6, TNFα, IL-12 and IL-23 (Chinetti-Gbaguidi, et al. 2015). More importantly, vascular injury break the balance between endothelium-derived cytokines and related inhibitors which attract SMCs (Otsuka, et al. 2012) and stem cells (Sata, et al. 2002) to the sub endothelium. The trapped cells become iSMCs under the influence of pro-inflammatory cytokines. It has been proven that the hematopoietic

stem cells have the ability to differentiate into different cells depending on the microenvironment. For example, under inflammatory condition stem cells selectively differentiate into the smooth muscle progenitor cells (Sata, et al. 2002). Studies using animal models demonstrate that endothelial dysfunction after a stenting procedure promotes bone marrow derived endothelial progenitor cell accumulation which promotes neointimal formation (Kipshidze, et al. 2004). Therefore, attempts to unravel the underlying molecular mechanisms of endothelial dysfunction may prove a better strategy for treating CVD in the future.

1.5.2 Macrophage

Macrophages regarded as phagocytic cells are widely exist in tissues and organs. Normally, activated macrophages act as an immune responder of the body's immune system to remove apoptotic cells and debris during the tissue remodelling process. Endothelial dysfunction initiates the inflammatory response and promotes accumulation of oxidized LDL in the intima of the vessel wall, which subsequently recruit the inflammatory cells like monocytes to arterial wall (Moore, et al. 2011). Once the monocytes migrate into the sub endothelial layer, they differentiate into macrophages which uptake oxidized LDLs in the intima. After excessive LDL uptake macrophages are converted into foam cells, another pathological type of the macrophage (Chistiakov, et al. 2017).

A variety of macrophage subsets with different protein expression profiles are found inside the atherosclerotic plaques. Based on *in vitro* investigations of cell markers and the production of the specific factors, macrophages can be classified into two major phenotypes, M1 pro-

inflammatory and M2 anti-inflammatory phenotypes. M1 macrophages are induced by TH1 cytokines such as TNFα and IFNγ, or interact with the lipopolysaccharide, meanwhile the M1 macrophage produces high levels of pro-inflammatory factors like IL-1β, IL-6, TNFα, IL-12 and IL-23 (Chinetti-Gbaguidi, et al. 2015). M2 macrophages can be further divided into three subclasses; M2a macrophages are induced by Th2 cytokines like IL-4 and IL-13, and M2b macrophages are polarized by treatment with the IL-1β plus some immune complex, IL-10, TGFβ1, while M2c macrophages are induced by glucocorticoids. All three subtypes of M2 macrophages produce high levels of IL-10 and TGFβ1, and low levels of IL-12 compared to the M1 macrophage (Chinetti-Gbaguidi, et al. 2015). Notably, macrophages maintain their plasticity and can switch from M1 to M2 phenotype depending on their environment (Porcheray, et al. 2005). *In vivo*, macrophages have a much more transient and heterogeneous expression and secretion profile due to a much more complex environment. Since many subtypes have been identified in atherosclerosis, it is clear that these macrophages could interact with each other, leading to a complex network in atherosclerosis lesions. Particularly macrophage subtypes play an important role in controlling inflammatory process. Take M1 and M2 type macrophages as an example, M1-like macrophages are found in both mice and human atherosclerosis and are regarded as pro-inflammatory macrophages because its role in promoting vulnerable plaques, whereas M2 macrophages have an anti-inflammatory character. One study found that in the ruptured plaque, both M1 and M2 macrophages populations increase, with M1 macrophages predominantly located in the most unstable regions of the atherosclerosis plaque (i.e the shoulder region of the plaque) while the M2 macrophages located far away from the lipid core of the plaque in the adventitia(Stoger, et al. 2012). Interestingly, foam cells

were observed to co-express M1 and M2 macrophages markers (Stoger, et al. 2012), raising question to the cellular origins of these cells (Porcheray, et al. 2005). Additionally, although the M1 and M2 macrophages were found to be located in different areas of the plaque, we still do not know whether this distribution occurs before or after macrophage polarisation. Finally, M1 and M2 macrophages were found to be equally distributed in the fibrous cap (Stoger, et al. 2012). Since the M2 macrophages have pro-fibrotic phenotype while M1 consistently promote the inflammatory activation, the fibrous cap might be the outcome of M1 and M2 competition and balance in this region.

Although SMC migration and proliferation in the intima and ECM accumulation are defining features of neointima formation and restenosis, inflammatory macrophage activation also plays a very important role in promoting neointima formation. EC injury or stenting implantation not only lead to the platelet activation and aggregation but also recruit the leucocytes like monocytes from the circulatory system. Leucocyte and platelet interactions play a key role in the initiation and progression of neoinitimal formation. Evidence indicates that monocyte adhesion is correlated with neointimal formation. Indeed, research shows that increased inflammation will induce more serious neointimal hyperplasia, and early chronic inflammation has a strong relationship with intimal thickening after stenting (Burke, et al. 2002). In a murine model of vascular injury- induced neointima formation, depletion of macrophages also results in the reduced neointima formation up to 30 days (Danenberg, et al. 2003). As discussed, macrophages release a set of cytokines, which contribute to SMC differentiation and proliferation, which are morphological predictors

of arterial remodelling in coronary atherosclerosis (Lv, et al. 2016). It is worth noting that other leucocytes, such as the neutrophils, also have an impact on intimal thickening. Administration a monoclonal Mac-1 antibody into a rabbit model was found to reduce neutrophil infiltration in the injured vasculature, which in turn decreased SMC proliferation (Welt, et al. 2000).

Bone marrow derived monocytes are the main source of macrophages in atherosclerosis. After initially being generated from hematopoietic stem and progenitor cells in the bone marrow, monocytes were first found to be resident in the spleen, where they undergo proliferation and activation before being released into the blood and recruited into blood vessel wall in the early stage of atherosclerosis (Tabas, et al. 2015). Quiescent monocytes generally stay in the spleen and lymph node, until induced by several chemokines released from the lesions. One study found that CCR2 (receptor of CCL2) deficient mice displayed lower levels of circulating monocytes, suggesting that CCL2 mediates monocyte release into the blood. It should be noted that other chemokines might play more important role in recruiting the cells to the lesions. For instance, abolishing CCL5 and CXCL1 in animal experiments directly reduced monocyte attraction to atherosclerotic lesions (Soehnlein, et al. 2013). Since endothelial dysfunction is the critical reason for monocytes accumulation in plaques, molecules such as the VCAM-1 and the P- selectin have also been demonstrated to play important roles in monocytes accumulation within atherosclerotic plaques. Although monocytes may contribute to a large number of macrophages in the plaque, other cells may also participate in macrophage accumulation. It is believed that although monocyte influx

takes place predominantly in early lesions processes, local macrophage proliferation accounts for most of macrophages accumulation in the advanced atherosclerotic plaques (Robbins, et al. 2013). One group identified a group of Sca-1 positive cells in the mice adventitia. These cells not only expressed the progenitor markers; CD34 and c-kit, but also leukocyte markers; Ly6C and CD45, suggesting that these cells may contribute to lesion macrophages and in turn affect atherosclerosis development (Psaltis, et al. 2012). Of note, intimal SMC phenotype switching was also reported to contribute to macrophages and foam cell population in advanced atherosclerotic lesion (Allahverdian, et al. 2014).

1.5.3 VSMC and iSMC

In circulatory system, VSMCs are the major cell types in the vascular tunica media. During the early stage of blood vessel development, SMCs resident in the medial layer of the vessel wall secrete a huge amount of ECM including collage, elastin, fibronectin, cadherin and integrin. Although ECM is not the primary element of the blood vessel, they provide the basic structure for the cells resident in the early stage of the vascular development (Owens. 1995). As the blood vessel matures, SMCs begin to undergo phenotype switching by reducing ECM secretion while increase intracellular myofilament expression to allow for contraction and dilation of the vessel wall, thereby regulating blood pressure and flow (Rudijanto. 2007). Because they are not terminally differentiated cells, VSMCs exhibit remarkable phenotypic plasticity in response to environmental stimulation such as vascular injury, inflammation, and lipoprotein accumulation. In the normal mature blood vessels, SMCs display a contractile state and act as a blood vessel diameter regulator to control the blood pressure and flow. In

this quiescent phenotype, SMCs express typical smooth muscle contractile proteins such as the SM-α actin, SM22α, SM myosin heavy chain(SM-MHC), H1 –calponin and smoothelin (Owens, et al. 2004). Under some pathological conditions, SMCs can undergo a phenotype switch from its contractile state to a synthetic state. The cells in this latter state are much more active, and can migrate into the intima and renew itself by undergoing proliferation. Besides, synthetic SMCs also produce ECM proteins, like the collagen and elastin, whereas at the same time the expression of smooth muscle contractile proteins is reduced. Researchers have shown that SMCs migrate and accumulate in the sub-endothelial layer after fatty streak formation and further develop a new fibrous cap in plaques(Gomez, et al. 2012). Another study found that in human atherosclerotic plaques, SMCs display chondrocyte-like phenotype with reduced expression of SMαA and increased expression of Sox-9 (Bobryshev. 2005).

During atherosclerosis, lipids and other triggers alter the endothelium which promotes circulating monocytes being transported into the SMCs layer and maturing into macrophages. Activated macrophage secrete growth factors as well as some inflammatory mediators, which promote SMC phenotype switching from quiescent state to synthetic state. Synthetic SMCs tend to migrate into the intima where they start to proliferate, produce extracellular matrix, and take part in fibrous cap formation. By determining telomere loss of the SMCs, scientists found that these SMCs from fibrous cap display monoclonal features with a higher level of replication compared with the cells in the medial layer. This demonstrate that the SMCs in the fibrous are not in the quiescent state and undergone proliferation when they participate into the atherosclerosis

development(Matthews, et al. 2006). Interestingly, increased SMCs in the cap of the atherosclerotic lesion was found to increase the likelihood of plaque stability during the atherosclerosis (Lee, et al. 1997), but the mechanisms for this are still unknown. Since SMCs lost contractile marker expression after phenotype switching, it is hard to identify the origin of smooth muscle-like cells in atherosclerotic lesion. Restenosis is the re-narrowing of the blood vascular after the revascularization procedures such as angioplasty and stenting. Followed the injury to the vessel wall, the platelets and inflammatory cells release multiple cytokines, which stimulate SMCs to proliferate and secrete ECM proteins in the intimal space, resulting in the intimal hyperplasia and narrowing the blood vessels (Chaabane, et al. 2013).

By using the genetic tools, studies have recently identified foam cells like SMCs expressing common macrophage markers in the plaque (Majesky 2016). One study found that ApoE$^{-/-}$ mice, with myocardin$^{+/-}$ gene background, display worse atherosclerosis with more macrophage and macrophage-like cells accumulation compared with control mice (Wang, et al. 2001). Since the myocardin regulates serum response factor (SRF) expression and VSMC contractile gene expression, loss of myocardin gene appears to induce the VSMC switch to a macrophage-like phenotype (Wang, et al. 2001). Another important phenotype is the iSMC phenotype generated from contractile SMCs undergoing phenotype modulation due to inflammatory cytokine co-stimulation. In the past 30 years, SMCs phenotype switching has been simply classified into the contractile and synthetic, however following huge progress in our understanding of the

molecular mechanism regulating SMC phenotypic modulation, it has become harder to define VSMC sub-phenotypes.

Monocytes recruited from the circulatory system produce cytokines such as TNFα and IL-1β, creating a local inflammatory environment for medial SMCs. One study found that IL-1β could mediate SMC phenotype switching into an inflammatory state by activating the NF-ƙB signal pathway *in vitro* (Alexander, et al. 2012). lSMCs typically become less contractile, show enhanced proliferation and migration with increased inflammatory cytokines production, take on a cobblestone-like appearance and participate into atherosclerotic plaque and neointima formation (Sobue, et al. 1999; Kawai-Kowase et al. 2007). One study shows that SMCs-derived TGFβ1 could mediate macrophage polarization *in vitro* and increase inflammatory chemokines expression (Ostriker et al. 2014). Histological experiments in animal models of the atherosclerosis showed that SMCs in the media of the vessel wall express a number of pro-inflammatory markers and inflammatory transcriptional mediators, including the VCAM-1(O'Brien et al. 1993), ICAM-1, CXCL2 and NF-ƙB (Alexander, et al. 2012; Landry, et al. 1997). In vitro experiments have also proven that SMCs producing these pro-inflammatory molecules increase monocyte adhesion and protect the cells from apoptosis (Cai, et al. 2004). Together the results show that there are a group of SMCs, which can express contractile markers of differentiation as well as the inflammatory genes under inflammatory conditions. This group of SMCs present a markedly different phenotype from the classical definition of SMC phenotypic modulation, whereby VSMCs in the contractile state express only contractile markers, and VSMCs in the synthetic state, mainly undergo proliferation and migration and

produce ECM proteins. Thus, iSMCs describe a type of SMCs present in atherosclerosis and restenosis, which display a similar phenotype to the synthetic/dedifferentiated SMCs, and co-express SMC proteins and inflammatory markers within the lesion.

Since atherosclerosis is regarded as a chronic inflammatory disease, it has been widely accepted that iSMCs play an essential role in atherosclerosis and neiointima formation. Multiple stimulatory factors in atherosclerotic lesions are considered to promote iSMC production. Low-density lipoprotein (LDL) accumulation and deposition within the blood vessel wall or intima due to hypercholesterolemia and dyslipidaemia is considered as a critical trigger of iSMC production (Alexander, et al. 2012). Meanwhile, endothelial dysfunction or injury is thought to provide a pro-inflammatory environment for iSMC production and accumulation. ECM alteration also induces SMCs to undergo pro-inflammatory phenotype switching (Orr, et al. 2009). Additionally, changes in blood pressure also affects SMCs phenotype switching by changing endothelial cell physiology (Mitchell, et al. 1999). According to the neointimal formation “response -to-injury” model proposed by Ross (Ross. 1993), SMCs of the tunica media contribute to the most part of the iSMCs in the neointimal hyperplastic process. However, growing evidence suggests that the medial SMCs contribution model might be too simple to account for the total SMC population in the atherosclerosis and neointima formation process, with other cells including ECs, circulating stem/progenitor cells and adventitial stem/progenitor cells discovered to be involved into iSMCs generation in neointimal formation and hyperplasia.

Apart from medial SMCs and stem/progenitor cells, additional cellular origin for iSMCs in the neointima are endothelial cells. ECs also exhibit a variety of phenotypes in the cardiovascular system. For instance, endocardium cells change into mesenchymal cells to form a part of the atrioventricular cushion. This occurs by a process known as 'endothelial-to-mesenchymal transition'(End-MT) (Eisenberg, et al. 1995). By investigating neointimal formation and hyperplasia after the grafting veins into the arterial circulation, a group found that endothelial lineage-derived cells experience End-MT and acquire a mesenchymal or SMC-like phenotype (Cooley, et al. 2014).

1.6 Stem and progenitor cells

Stem cells are group of cells which have permanent potential to self-renew and differentiate into multiple types of cells, while the progenitor cells have limited capacity to differentiate into specific cell lineages related to its location. The exact definition for stem and progenitor cells remains debatable. It has been suggested that stem cells and progenitor cells are similar cells but present at different stages, with stem cells losing their stem cell characteristics and gradually becoming progenitor cells before committing to a mature cell phenotype. To make it simple, we termed these cells as stem/progenitor cells (SPCs). The SPCs in the adult body are a mixed pool of cells, resident in specific harbours called "SPC niches", and exist in a variety of tissues and organs like the bone marrow, the peripheral blood, skin and the vessel wall. Accumulating evidence has suggested that these SPCs play an important role in atherosclerosis and restenosis, with the circulating and vascular wall resident SPCs implicated in various vascular diseases.

1.6.1 Circulating Stem and progenitor cells

Mesenchymal stem cells (MSCs), one type of multipotent stem cells, were believed to be mainly derived from the mesenchyme, but more and more studies have shown that these cells could be identified and isolated from almost all the tissues/organs including the neuroepithelium and neural crest. Due to their multipotencies, MSCs can either differentiate into a mesodermal lineage or give rise to non-mesenchymal tissue both *in vivo* and *in vitro*. It has been shown that these cells participate in vascular neointimal formation and restenosis. Another type of circulating SPCs is endothelial progenitor cell (EPC) that was first reported in 1997 (Asahara, et al. 1997). EPCs can be isolated from bone marrow and peripheral blood, and contribute to *in vivo* angiogenesis. Five years later, a study reported a novel type of SPCs with the capacity to differentiate into SMCs with specific growth, adhesion and integrin profile exist in human blood, classified as smooth muscle progenitor cell (SMPCs) (Simper, et al. 2002). Another group also succeeded in isolating these SMPCs from human peripheral blood, which express SMαA, as well as CD14 and CD105. Following long-term culture, these SMPCs display a contractile phenotype and express typical SMC markers (Sugiyama, et al. 2006). Interestingly, CD14 negative SMPCs, which produced less proteases and inflammatory cytokines than that of VSMCs was reported in a later study (Simper, et al. 2010). Though these SPCs were proven to exist in the circulating system and have the ability to differentiate into the smooth muscle-like cells *in vitro*, debate remains over whether these cells participate in atherosclerosis and restenosis. By tracing these bone marrow derived SPCs with the eGFP, Bentzon and his group found that no cells, differentiated from the bone marrow cells, were in the atherosclerotic plaques of a mice (Bentzon, et al. 2006). Later in another

model of plaque disruption model, this group also demonstrated that only SPCs resident in vessel wall participate in the healing process (Bentzon, et al. 2007). These data infer an important role for the local SPC population in atherosclerosis and restenosis.

1.6.2 Adventitial stem and progenitor cells

In the past decades, many studies have reported that there are large number of SPCs in the vessel wall. Although debatable, it has been widely accepted that the stem cell niches in the sub endothelial layer harbour variety of SPCs. As aforementioned, the tunica adventitial is the outside layer of the vessel wall which composed with connective tissue, terminal nerve fibres, collagen and fibroblasts as well as some quiescent inflammatory cells such as the tissue macrophages (Gutterman. 1999). For decades, the adventitia was considered to be 'quiescent', and have little to no involvement in vessel wall homeostasis. Now, scientists believe that cells in the adventitia layer play more than just a structural role in vascular function. The cells in the adventitia, such as adventitial fibroblasts were reported to contribute to the sub-intimal proliferation during the atherosclerosis. Moreover, cells from the adventitial have also been shown to participate in vascular calcification in the chronic atherosclerotic lesions. Additionally, neurotransmitters released by the nerve in the adventitial could play a role in regulating the vessel tone (Gutterman. 1999). More importantly, immunohistochemistry assays found that large number of progenitor cells were present in the adventitia of the vascular wall and positively expressed SPC-like markers; Sca-1, c-kit, CD34 and FLK-1 (Campagnolo, et al. 2010; Worsdorfer, et al. 2017; Hu, et al. 2004). These

SPCs could differentiate into SMCs in response to injury in an attempt to repair the damaged tissue.

Specifically, Hu et al reported that these SPCs express Sca-1, CD34, c-Kit, Flk1 but no the SSEA-1 as the cell surface marker, and differentiate into SMCs *in vitro* in response to PDGF-BB. After transfered to the adventitial side of the vein grafts ApoE$^{-/-}$ mice, these cells could migrate through the media and enhance atherosclerotic intima formation (Hu, et al. 2004). Later, another study found that these Sca-1^{+} SPCs were located closely to the vasa vasorum and displayed the potential to differentiate into SMCs, adipocytes, osteoblast and chondrocytes *in vitro*. When these cells were implanted onto the outer layer of vein grafts of ApoE$^{-/-}$ mice, of the authors observed about 30% of neointimal SMCs were derived from transplanted SPCs (Chen, et al. 2013). Another pre-clinical study showed that sirolimus induced Sca-1^{+} SPC migration and differentiation into SMCs in the intima, which promoted restenosis (Wong, et al. 2013). This thesis indirectly proved that these adventitial SPCs were involved into the neointima formation as well as restenosis. Interestingly, the origin of these Sca-1^{+} cells has led to some suggestions that some of them are derived from mature SMCs. Using two different genetic-fate mapping methods to trace the Sca-1 progenitor cell ancestors *in vivo*, about 8% of Sca-1^{+} cells were shown to come from the mature SMCs of the medial layer, while no cells were derived from the peripheral blood mononuclear cells s (Majesky, et al. 2017). Another group of CD34^{+}/CD31^{-} progenitor cells were also found in saphenous veins, and were negative for the endothelial markers; vWF and CD31, but positive for progenitor cell markers; C-Kit and CD133 (Campagnolo, et al. 2010). Notably, a few of these cells were also found to be positive for NG2 and PDGFRβ

markers, which means these cells may have some relation with MSCs. Further experiments using these cells demonstrated that $CD34^+/CD31^-$ cells were able to self-renew and differentiate into mesodermal lineages, although they failed to differentiate into ECs. After a few years, another group also succeeded in isolating $CD34^+/CD31^-$ $/CD146^-/CD45^-$ cells from human stromal adipose tissue around the vascular adventitia (Corselli, et al. 2012). These cells displayed MSC-like features and could be induced to differentiate into pericyte-like cells *in vitro*. Moreover, one study focusing on cell origin during the restenosis reported that a population of $NG2^+$/ $CD146^+$ cells exist in the vascular adventitial, which contribute to the restenosis following arterial injury (Tigges, et al. 2013). All these studies show that these adventitial SPCs do exist and contribute to the SMC accumulation during the restenosis after the arterial injury surgery.

Another group of progenitor cell were found in a region called 'vasculogenic zone', located between the medial and adventitial layer of the human blood vessel. These SPCs also participate in vascular structure formation in a transplantable tumor model *in vivo* (Zengin, et al. 2006). Further investigations were able to divide these cells into two groups which are $CD34^+$ / $VEGFR2^+$ $/Tie^+/CD31^-$ /cells and $CD34^-/CD3^-$ $/CD44^+/CD90^+/CD105^+/CD73^+$ cells, with the former group of cells though to differentiate into the ECs, whilst the latter group are thought to differentiate into SMCs(Tilki, et al. 2009). Another study also reported similar findings (Torsney, et al. 2011), demonstrating an ability of these progenitor cells to differentiate into the multiple mature cells in selective culture media *in vitro*. MSCs were defined with cells as being able to differentiate into a variety of cell types with minimal immune-response

gene expression profiles. It is ruled by the international cell therapy society published in 2006 that the MSCs were defined by $CD105^+$, $CD73^+$ and $CD90^+$, but $CD45^-$, $CD34^-$, $CD14^-$ or $CD11b^-$, $CD79a^-$ or $CD19^-$ as well as $HLA\text{-}DR^-$ (Dominici et al. 2006). By this definition, one group have identified a MSC population ($CD44^+$/ $CD73^+$ /$CD34^-$/$CD45^-$) in adult human arterial adventitia which present typical MSC characteristics and exhibit multi-potency (Klein et al. 2011). Notably, these cells were able to be induced to differentiate towards SMCs under TGFβ1 treatment *in vitro*. However, contradictory results by other groups found that $CD34^+$ cells in the vasculature also display an MSC-like character. One study discovered a group of $CD34^+$/$CD31^-$ cells from human saphenous vein. These cells expressed mesenchymal antigens such as CD44 and CD90 as well as the stem cells marker SOX2, but not the endothelial marker; Von Willebrand factor (VWF). Moreover, these cells displayed multiple differentiation capacity *in vitro*, and were able to give rise to osteoblasts, adipocytes, and myocytes (Campagnolo, et al. 2010). These data suggest that MSCs are mixed and heterogeneous populations with different origins, which make it more difficult to direct compare their functions and contributions to disease pathology. Despite this, it has become clear that these MSCs are able to respond to vessel injury and are involved in several biological processes in the vasculature. One study shows that in response to vessel injury, adventitial cells are likely to migrate through the tunica media and into the intimal site and acquire a SMC-like phenotype resulting in enhanced neointimal formation (Kramann, et al. 2016), indicating that MSCs in the adventitial layer are another candidate for iSMC accumulation in the neointimal lesions.

1.6.3 Sonic Hedgehog (Shh) signalling pathway

Shh signalling pathway also known as Sonic hedgehog-glioma-associated oncogene 1 (Shh-GLI1) signalling and Hedgehog-patched smoothened (Hh-Ptch- Smo) signalling pathway, is one of the major signal networks which plays an important role during the embryonic development as well as the cells differentiation. The Shh signalling pathway is a highly conserved signal transmission pathway which is frequently active during the development for intercellular communication. In adult tissues, Shh signalling is quiescent or poorly active unless some specific stimulation present, for instance, it was reported that Shh signalling was active during wound healing (Le, et al. 2008). Many researchers discovered that Shh signal pathway is widely recognized to participate into the development of various tumours as well as in tumorigenesis (Katoh, et al. 2009) and embryonic development in diverse organisms (Taylor, et al. 2002).Moreover, Shh-GLI1 signalling has been extensively implicated in adventitia smooth muscle progenitor cell self-renewal and differentiation (Majesky, et al. 2012; Passman, et al. 2008) and blood vessel formation (Kolesova, et al. 2008). $Shh^{-/-}$ mice were reported had less number of $Sca\text{-}1^{+}$ cells in artery (Koleva, et al. 2005). This suggest Shh signalling pathway plays a critical role in AdSPCs and may involve in the SMCs differentiation. Shh signalling can be activated though two major ways: canonical signalling and non-canonical signalling (Carballo, et al. 2018). Canonical signalling activation start with Shh binding to the cells membrane receptor 12-ransmembrane protein Patched (Ptch1), once Ptch1 interacted with the Shh its inhibitory function of a Smo, a G-protein-coupled receptor like protein, is abolished. Cytoplasm accumulation of Smo will cause the translocation of GLI family protein into the nucleus, which triggers target gene transcription. The non-canonical Shh signalling is mediated through GLI independent way. The first one is though Smo

modulated-Ca^{2+} influx. The second one is directly interacting, activating cyclin B1, leading to cell proliferation and survival (Carballo, et al. 2018). Shh signalling pathway could interact with other pathways such as Wnt pathway to control stem cells differentiation. Many components could participate in and regulate Shh signalling activation. Supressor of Fused (Sufu) has been considered as a negative regulator of the Shh signalling pathway. It was said that Sufu could regulate the GLI1 and GLI2 activation at several levels (Murone, et al. 2000; Dunaeva, et al. 2003). Moreover, some studies found that Sufu could negatively regulate Shh signalling through direct binding with GLI1 and retaining GLI1 in the cytoplasm(Dunaeva, et al. 2003; Kogerman, et al. 1999).

1.7 RNA interference and small noncoding RNAs

Multiple mechanisms include DNA/RNA modifications, transcription, post-transcription and translation control a given gene regulation. Among them, RNA interference (RNAi) is an extensive mechanism used by all eukaryotic cells to regulate gene expression via mRNA degradation, translation suppress and chromatin remodelling (Zotti, et al. 2018).

1.7.1 Small noncoding RNAs

Small noncoding RNAs is the primary class of RNA in RNAi process, which is usually about 20-30nt in length and play a negative regulate role during the gene expression (Ghildiyal, et al. 2009). Because of their origin, structure and the role in specific biological processes the small non coding RNAs are classified into three classes: genome encoded microRNAs (miRNAs) (Lambert, et al. 2019), small interfering RNAs(siRNAs) (Zamore, et al. 2000), Piwi- associated small RNAs(piRNAs) (Brennecke, et al. 2007).

1.7.2 Small interfering RNAs (siRNAs) biogenesis

Small interfering RNAs (siRNAs), first discovered from plants and later from other species, is transcripted and matured from a longer double –stranded RNA, similar to miRNAs. Both miRNAs and siRNAs are cleaved by an RNase III enzyme called Dicers, despite that they have very different structure. Further research in drosophila found that Dicer family contains two different gene called Dicer-1 and Dicer-2. Later study reported that Dicer-1 is necessary during the miRNA mature, while Dicer-2 is required in dsRNA processing into siRNAs (Lee, et al. 2004). siRNAs degrade or inhibit the target gene expression by forming a RNA-induced silencing complex (RISC) with ribonucleoproteins (Hammond, et al. 2000). The RISC interacts with the antisense strand from the siRNAs to form an active RISC complex. The left passenger strand was cleaved by the endonucleases argonaute 2(AGO2). After binding to the cognate mRNAs the active RISC complex cleavages and thus inhibits the target gene expression(Pecot, et al. 2011)(Figure 3).

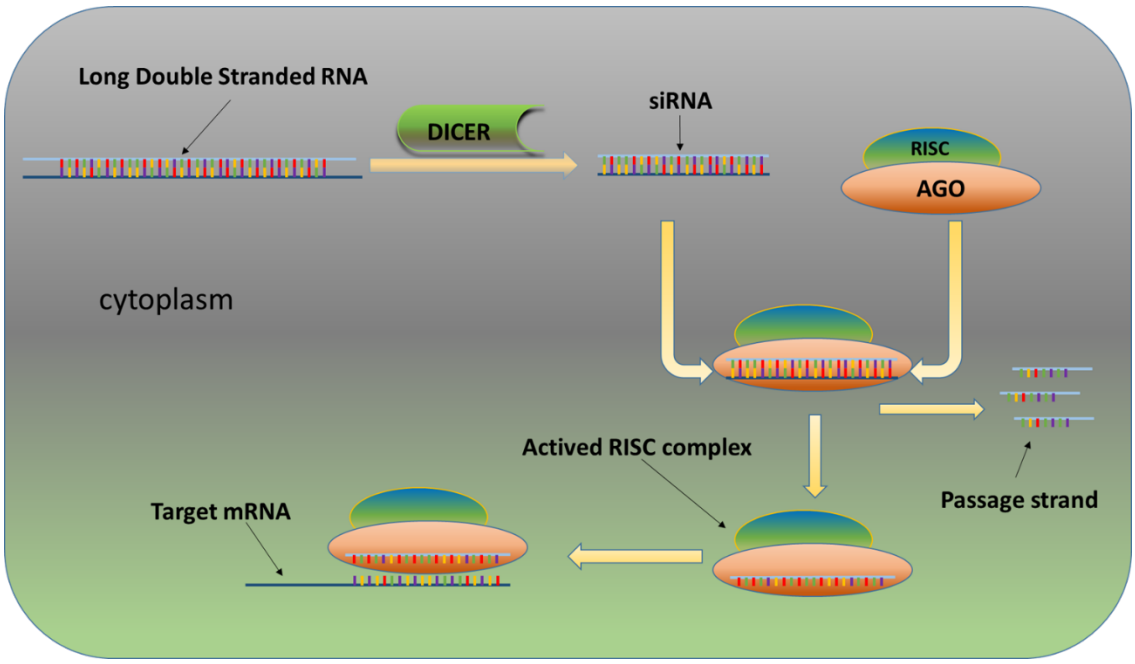


Figure 3. SiRNA regulate the gene expression. The ds-RNAs were processed by the Dicer and become ds-siRNA, these siRNAs were loaded into RISC and AGO complex. The passage strand were cleaved into small pieces, while the guide strand incorporate into RISC complex and lead to cleavage/supress the target mRNA.

1.7.3 Piwi-interacting RNAs (piRNAs) biogenesis

Piwi-interacting RNAs (piRNAs) are the most recently discovered small RNAs. It was first discovered in Drosophila in 2001(Aravin, et al. 2001). Later studies in other species like fish, files and mammals showed these small RNAs were especially associate with Piwi clade of Argonaute protein (a sub-class of Animal Argonaute protein), and named as Piwi-interacting RNAs (piRNAs). The piRNA is the longest small noncoding RNAs with a length of 25-30nt.PiRNAs can be distinguished from siRNAs or miRNAs since they are restrictedly binding with Piwi proteins and do not need Dicer for their maturation.

The biogenesis and maturation of piRNAs is still not well understood. Some studies suggest that piRNAs are cleaved from the coding and 3'untranslate regions of the single-strand precursor RNAs (Vagin, et al. 2006), which is

different from siRNAs and miRNAs. The mature piRNAs have a phosphate at its 5' terminus, and similar structure to siRNAs in flies at 3'termini (Kirino, et al. 2007). The piwi proteins differ across different species, for instance in mice, the piwi family are comprised of MILI, MIWI and MIWI2, while in human HILI, HIWI1, HIWI2 and HIWI3 are classified as the piwi family proteins (Ghildiyal, et al. 2009). Two major pathways, primary pathway (phased piRNAs pathway) and secondary pathway (Ping-pong cycling) (Czech, et al. 2016), have been proposed to control/regulate the production and maturation of piRNAs. In the primary pathway, a genomic region called piRNAs clusters are transcribed into a long single stranded RNAs. Interestingly, not all the mRNA transcripts could produce piRNAs, and only 5' mono-phosphorylated RNAs, a structure is critical for Argonaute protein binding, can produce the piRNAs. Once the single strand RNA interacted with the PIWI proteins, the RNA transcripts are modified at 3' terminus before they become mature piwi-RNAs (Ozata, et al. 2019). In the 'ping-pong' cycle or secondary pathway, the piRNA- PIWI protein complex acts as a cleavage director, which ultimately generate a new piRNA. First, the piRNA cluster transcripts are recognized by Argonaute3 protein and cleaved to piRNA intermediates. These intermediates are loaded into Aubergine (Aub) protein. After then, two nonexclusive ways lead the piRNA to different destination. One is cleaved by Zuc at 3' end and associated with PIWI proteins, producing a mature piRNA. In another way, the 3' end subjects to Papi-dependent trimming and Hen1 mediated methylation. The methylated piRNA- Aub complex is then modified by dimethyl -arginine (sDMA), and later recruited into Krimper -Ago3 protein complex. After interacted with Krimper the piRNA were loaded again into Ago3. These Ago3-loaded piRNA intermediates are either subjected to cleavage by Zuc or trimming and methylation by Hen1 for their maturation. The matured

Ago3- piRNA complex are then used as a pre-piRNA precursor for another cycle. Notably, mature piRNAs produced by this pathway always contain a 10-nt complementarity sequence to the director piRNA (Ozata, et al. 2019)(Figure 4).

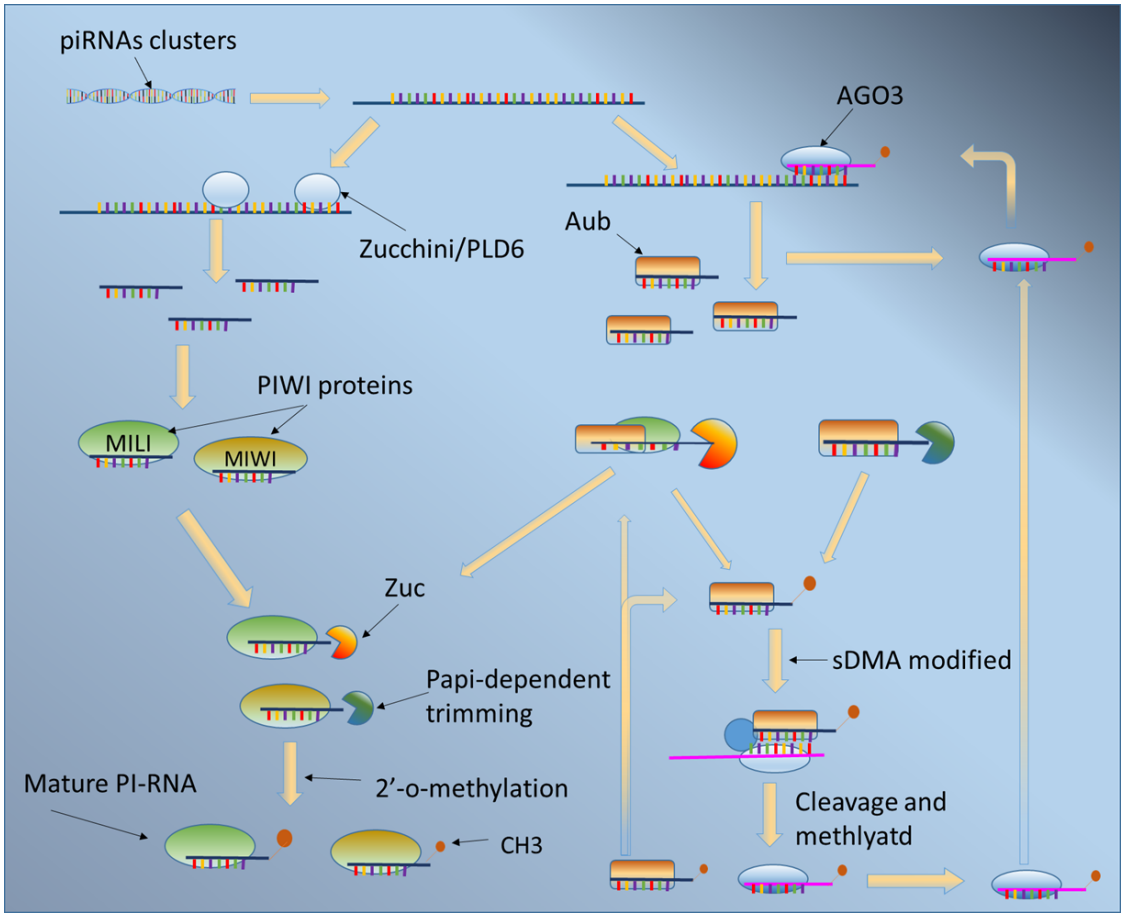


Figure 4. Piwi-RNA biogenesis. Two different pathways were reported to be involved in piRNA production and maturation. Left illustrating primary pathway, while right part describing the 'ping-pong' cycling. In the primary pathway, the long precursor transcripts RNA is cleaved by a mitochondrial protein, Zucchini/PLD6. After bound to a PIWI protein, the 3'end of the pre-RNA were modified by PNLDC1, a single stranded RNA exonuclease, followed by methalyation by Hen1/HENMT1 at the 3'end, producing mature piRNA. The 'ping-pong' cycle is much more complicate, AGO3 protein binding is associated with cleavage of the piRNA precursor transcript, while Aub protein binding is a critical step for transporting the pre-piRNA within the 'ping-pong' cycle (Ozata, et al. 2019).

The function of piRNAs and Piwi proteins is still not well studied, some research suggest piRNAs may play a role during the meiosis (Li, et al. 2013). Mammalian piRNAs possess much more complicated functions than their counterparts in flies. There are two major mammalian piRNAs, pre-

pachytene and pachytene piRNAs (Aravin, et al. 2007). Apart a role in meiosis, piRNAs have also been implicated in protecting the germline double-strand DNA breaks (Aravin, et al. 2001). piRNAs have also been reported to mediate post transcriptional transposon silence (Lewis, et al. 2018; Reuter, et al. 2011). The regulatory mechanisms of given piwiRNA vary among different species. For example, in flies, piRNAs recruit the dSetdb1 and then promotes H3K9 methylation (Wang, et al. 2011), but it is unclear if the mouse nuclear PIWI protein MIWI2 could promote DNA methylation. Although the piRNAs was first reported to protect the germline cells, their functions in other tissue and cells were also observed. For instance, piRNAs play important roles in stem cell maintenance and regeneration in melanogaster (Palakodeti, et al. 2008), and in mosquito the somatic piRNAs exert a protective effect in the viruses infection(Miesen, et al. 2015).

1.7.4 MiRNAs biogenesis

MiRNAs are a group of highly conserved and endogenous non-coding RNAs which were first recognized in 1993 (R. C. Lee, et al. 1993). The lin-4, as the first identified miRNA, was proven without any protein coding potntial but to inhibit the target gene, lin-14, by direct interacting with the 3' un-translation region (3'UTR). (Wightman, et al. 1993). Since then, miRNAs are extensively identified in different species, including plants and animals (Bartel,et al. 2004).

MiRNA biogenesis processes are starting with primary (pri-miRNA) miRNA transcription from miRNA genes by RNA polymerase II (Pol II). The pri-miRNA transcripts containing a distinctive hairpin structure is loaded into

protein complex of Drosha, a type III RNase, and protein DGCR8, within which the pri-miRNAs are subjected to multiple cleavages at their 5'and 3' terminals to produce pre-miRNA (miRNA precursor). After loaded into exportin 5-RAN/GTP complex the pre-miRNAs are transported from the nuclear to cytoplasm, where the RNase III Dicer and TAR RNA binding protein (TRBP) work cooperatively to remove the loop from pre-miRNA, resulting in a double-strand miRNA (ds-miRNA). The resulting ds-miRNAs are loaded into argonaute (AGO) family of proteins, which unwind ds-miRNA duplex. After then, the guide strand incorporates into the core of the RNA-induced silencing complex (RISC), forming a mature miRNA regulate complex, and the passenger strand is transported into P-body and degraded(Rupaimoole, et al. 2017) (Figure 5). However, recent data shows that the passenger strand also has the same function as the guide strand (Schober, et al. 2014).

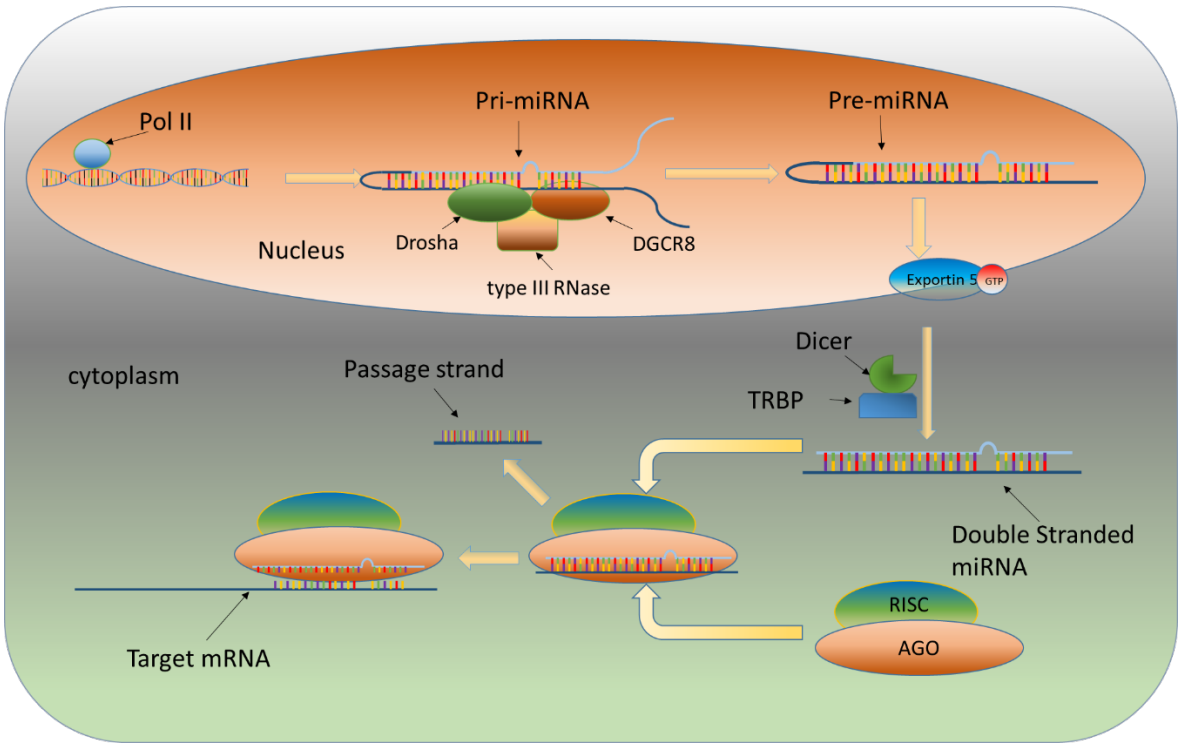


Figure 5. MiRNA regulates the gene expression. The pri-RNAs are transcript by polymerase II (Pol II) enzyme from genome, and then interacted with the Drosha complex to produce pre-miRNA. Pre-miRNAs are transported from the nuclear to cytoplasm by Exportin5-RAN/GTP complex. In cytoplasm, pre-miRNA is processed by the Dicer to generate ds-miRNA, which is unwound within RISC and AGO complex to produce mature miRNAs. The passage strand is cleaved into small pieces within P-body, while the guide strand reloads into RISC complex and binds to the target mRNA, supressing the target gene expression.

SiRNAs and miRNAs are very similar in structures and biogenesis, and they both could inhibit target gene expression, but through different mechanisms. The siRNA requires for full complementary to its target mRNA to trigger the target mRNA degradation by different exonucleases. Contrastingly, such a requirement is unnecessary for miRNA to supress its target gene expression. The first 2-8 nucleotides of miRNAs, recognised as 'seed sequence', is critical for target gene regulation. To regulate target gene expression, miRNAs usually bind to one or more miRNA binding sites located within the 3'untranslated region (3' UTR) of target mRNAs through their 'seed sequence'. However, later studies also suggest that miRNAs can also regulate target gene expression by interacting with the binding site(s) outside 3' UTR, such as code region and 5' UTR (Valencia-Sanchez, et al.

2006). Since the 3'UTR of each gene contains binding site(s) for multiple miRNAs, thus each gene could be regulated by several miRNAs. Meanwhile, each miRNA could regulate different target genes.

Two mechanisms for miRNA-mediated gene regulation were reported: decaying/degrading mRNAs or repressing the translation efficiency of target genes. The former is mediated through endonuclease cleavage of the target mRNAs when the 'seed sequence' is perfectly matched with its binding site(s), while the latter is mediated through silencing/impairing the mRNA translation there is an imperfect matching between miRNA and target mRNAs. However, one study showed that miR-196 could mediate HOXB8 mRNA decay without perfect pairing, suggesting that perfect matching between miRNA and its target mRNA is not compulsory for the mRNA cleavage induced by some miRNAs (Yekta, et al. 2004). Another paper reported that the Argonatue protein 2 (AGO 2), the only one in the Argonatue family, is required for miRNA-RISC complex to catalyse mRNA cleavage (Meister, et al. 2004). On the other hand, it was first observed that lin-4 meditates the lin-14 protein amount but rarely changes the mRNA quantity(Lee, et al. 1993), indicating that silencing/impairing mRNA translation may come to play, which is largely supported by later studies. For instance, miR-15a and miR-16-1 influence the protein Bcl2 expression level, but have no effect on Bcl2 mRNA level (Cimmino, et al. 2005). Let-7 was reported to carry its target mRNAs to polysomes, and consequently represses the mRNA translation. Additionally, the mRNAs could be brought by miRNA to the P-Bodies (PBs) for storage, which suggest that the PBs is not only the site for mRNAs degradation, but could act as a ware house for storing the repressed mRNAs (Pillai, et al. 2005).

1.8 MiRNAs and diseases

More than 1900 miRNAs were discovered so far (miRBase/ http://www.mirbase.org/cgi-bin/browse.pl). MiRNAs participate in almost every biological process and sometimes act as a critical regulator of controlling cell proliferation, death, migration, differentiation and survival. Animals absent of miRNAs display embryo development defect in early stage (Wienholds, et al. 2003). Another seminal paper showed that during the mice embryo development, Dicer1 gene inactivation could induce the embryos died at early stage, means that miRNAs as well as other small RNAs produced by Dicer1 are critical for maintaining the stem cell amount and functions in mouse embryo development(Bernstein, et al. 2003). One study with embryonic stem cell (ESCs) showed that miR-214 could promote ESC differentiation into SMC-like cells (Wu, et al. 2017). The let-7 family of miRNAs could promote ESC differentiation and maintain the cells mature statues, while opposite effect was observed with ESCC miRNA families. (Melton, et al. 2010). Together, these studies suggest a divergent role for any given miRNAs in regulating ESC self-renew and differentiation. Similarly, huge number of miRNAs have been implicated in almost every human disease, particularly cancers and CVDs.

1.8.1 MiRNAs in cancers

Cancer is one of the leading causes of death. Progressive accumulation of the genetic and epigenetic alterations of oncogenes and some tumour suppressor genes allow the cells to obtain new properties, such as uncontrollable cell proliferation and migration. Those are two main underlying causes for cancer development and deterioration. The

processes for cancer formation and development divide into five steps: trigger, promotion, malignant transformation, progression and metastasis(Zhang, et al. 2007). MiRNAs have been reported to be involved in all these steps. One of the early studies reported that two miRNAs (miR-15 and miR-16) were down-regulated or missing in chronic lymphocytic leukemia (CLL) cells and CLL patients (Calin, et al. 2002). B cell lymphoma 2 (Bcl2) plays an anti-death role in eukaryotic cells, and increased level of Bcl2 protein was observed in many types of cancers, such as leukaemia and lymphoma. Both miR-15a and miR-16-1 were found to reduce Bcl2 protein expression in B cell CCL and increase apoptosis of the cells isolated from CLL patients (Cimmino, et al. 2005). Lung cancer is another common cancer in human. One study observed decreased level of let-7 miRNA in human lung cancer cells both *in vivo* and *in vitro*. This thesis also reported that lower let-7 expression in lung cancer patients have a worse prognosis and lower survival rate (Takamizawa, et al. 2004). RAS is a well-recognized oncogene. One study found that all three RAS genes were negatively regulated by let-7, and lung tumour tissues express a high level of RAS gene but lower level of let-7 gene (Johnson, et al. 2005). Other studies showed that miR-17-92 cluster was upregulated in lung cancer cells (Hayashita, et al. 2005), and miR-372 and miR-373 could promote testicular germ tumour cell proliferation and tumour process by targeting P53 signalling (Voorhoeve, et al. 2007). Since miRNAs could target multiple genes, they are not always function as an oncogene or tumour suppressor in diseases. Take miR-26 as an example, one research found that miR-26a expression was dramatically increased in glioblastoma multiform (GBM) patients, and phosphates and tensin homolog (PTEN)/ Akt signalling pathway was responsible for miR-26's oncogenic functions in glioma(Huse, et al. 2009). However, an opposite effect was observed in human thyroid carcinomas. By using two thyroid

carcinomas cell lines FB-1 and FRO, the authors showed that miR-26a and miR-125b could effectively reduce the cell proliferation. These result suggest this two miRNAs might negative regulate thyroid cell proliferation and present them as tumour suppressor genes in human thyroid carcinomas (Visone, et al. 2016). In short, miRNAs play a distinct role in different cancers, thus more investigation into the functions of these miRNAs could provide a new method to predict the patient's survival rate and treat cancer by modulating the specific miRNAs. For example, MRX, act as a miR-34 mimic, could inhibit multiple oncogene signal pathway and induce the tumour cell apoptosis, and has now been used in clinical trial for treating the primary liver cancer (Farooqi, et al. 2016). Any promising data rasied from such a trial would provide a new avenue for us to use miRNAs as a novel weapon for fighting cancers.

1.8.2 MiRNAs with other diseases

Cells or tissue dysregulation predispose disease progression, and miRNA dysregulation may play an important role in this initiate step. Neurodegenerative disorders (NDDs) are a group of neuronal degenerative diseases, characterised as a certain proportion of neuron undergoing progressive loss/damage in nervous system (Karnati, et al. 2015). As a powerful gene regulator, miRNAs have unsurprisingly been reported to play many critical roles in NDD. Take Alzheimer's disease (AD) as an example, Hebert et al observed an increased expression level of BACE1 gene, but decreased levels of miR-29a and miR-29b-1 in AD patients. The authors further confirmed that BACE1 is one of the target genes of miR-29 subgroup, suggesting that dysregulation of miR-29 subgroup may cause increased BACE1 protein expression and contribute to AD process (Hebert, et al. 2008).

Another research showed that miR-133b level was significant decreased in rat brain after Stroke compare with normal group, and the impaired expression of miR-133b was reversed by administration of MSCs. Additional evidence suggest that miR-133b released by MSCs could transfer to brain parenchymal cells and promote neurite outgrowth, which in turn improve neuron growth after Stroke(Xin, et al. 2012).

Even nowadays, virus infection still is one of most terrifying diseases. Virus infection happens in people's everyday life, causing from influenza to death. Hepatitis C is one the widely reported virus infection, which is caused by hepatitis C virus (HCV). After infected, HCVs trigger the liver chronic inflammatory process, leading to liver fibrosis and hepatic carcinoma (Manns, et al. 2017). Many miRNAs have been implicated in HCV infection or hepatitis C. Among them, the liver-specific miR-122 caught the researchers' attention. One research group found that the HCV viral replicon RNA level was significant decreased miR-122 inhibition. Further investigation revealed that miR-122 could directly interact with HCV RNA by binding to its' 5' UTR, resulting in HCV genome stabilization and increase of the replication of HCV in the liver cells (Jopling, et al. 2005). This research implied that the liver-specific miR-122 could be a therapy target in controlling HCV infection. Actually, as we all know targeted therapy could reduce the drug toxicity in patients and improve the therapeutic effect, miR-122 would be a perfect drug target for treating HCV infection. For such a purpose, a drug called Miravirsen, a high affinity anti-miRNA drug for miR-122, has been recently developed. Animals experiment with HCV-infected chimpanzees reported that Miravirsen could effectual suppress the miR-122 expression *in vivo* and significantly decrease the HCV RNA level after

two-week treatment. More importantly, HCV was undetectable after treated for 12 weeks(Lindow, et al. 2012). Following these promising data generated from pre-clinical studies, Miravirsen has now become the first miRNA-target drug used in phase II clinical trials, in which the investigators observed that Miravirsen showed a dose dependent effect when used in patients with genotype 1 HCV infection, with subcutaneous injection a higher dose for 5 weeks resulting in lower or even undetectable HCV RNA levels (Janssen, et al. 2013).

Diabetes mellitus (DM) is another very common but serious metabolism disease worldwide. Patients with DM is rapidly increased in the past three decades, and will continue to increase, with an estimate of 439 million DM, as much as 7.7% of world aged 20-79 years old people in the world in 2030(Chen, et al. 2011). Many studies have reported a critical role for miRNAs in DM, and suggested that these newly identified miRNAs could provide a new avenue for controlling or treating diabetes(Bhatia, et al. 2015). Just list one as example, one study reported that miR-103 and miR-107 could regulate the insulin sensitivity, and over-expression of miR-103 and miR-107 could induce a dysregulated glucose homeostasis in mice (Trajkovski, et al. 2011). The authors further identified Cav1, a regulator of insulin receptor, as one of the target genes for these two miRNAs (Trajkovski, et al. 2011).

1.8.3 MiRNAs in cardiovascular diseases

As mentioned previously, CVD is one of the leading causes of morbidity and mortality in the world, especially in developed countries. Since discovered, these studies have provided clear evidence to support that miRNAs are the

key regulators involved in almost all the biological processes, human diseases particularly CVD (Wu, et al. 2017; Afzal, et al. 2016; Yang, et al. 2018) as well as embryo development (Wienholds, et al. 2003; Bernstein, et al. 2003). Handful of miRNAs have been implicated in cardiovascular system development. For instance, a cardiac specific mRNA, MiR-208, produce from the intron region of human and mice α-myosin heavy chain (αMHC) genes, has been identified as a key miRNA to control the heart functional response to stress and hypothyroidism (van Rooij, et al. 2007). Specifically, the authors found that although the animal with miR-208 loss did not display any obvious abnormal phenotype in terms of heart development, these mice lost their ability to develop a cardiac adaptive response to pressure overload (van Rooij, et al. 2007). Other miRNAs, miR-133 and miR-1 have also been reported to play a role in controlling cardiac hypertrophy (Care, et al. 2007).

Apart from cardiac hypertrophy, numbers of miRNAs show a cell/tissues-specific expression pattern and have been implicated in cardiovascular system development and angiogenesis. The first miRNA has been assigned a role in these processes is the EC specific miRNA, miR-126 (Nicoli, et al. 2010). In this zebrafish, miR-126 induced by the flow could active the VEGF signalling by repressing spred1, the VEGF signalling inhibitor, and then promote angiogenesis (Nicoli, et al. 2010). In the absence of miR-126, zebrafish display severe embryonic developmental defects due to lacks of vessel integrity and calibre. Interestingly, spred1 overexpression could mimic the observed phenotypes in miR-126-deficient zebrafish (Fish, et al. 2008). On the other hand, the SMC-specific miRNA, miR-145 has been recognised as important regulator for SMC development. In this aspect,

miR-145 was first reported to promote SMC differentiation from fibroblasts (Cordes, et al. 2009). Serum response factor (SRF) is the master regulator for SMC differentiation and its phenotype switching. Both miR-143 and miR-145 expression were induced by SRF activation, suggesting that these two miRNAs are the transcriptional downstream targets of SRF signalling, and they could play important roles in SMC biology controlled by SRF (Xin, et al. 2009). MiR-145 deficient VSMCs stopped at synthetic state and less neointimal lesion was observed in miR-145 mutant mice (Boettger, et al. 2009). Apart being a critical regulator for cardiovascular system development and angiogenesis, growing evidence has also suggested that miRNA dysregulation is the main underlying cause for almost all the CVDs such as atherosclerosis, heart failure and hypertension.

Atherosclerosis is a chronic inflammatory diseases of vascular wall starting with EC injury and dysfunction (Rudijanto. 2007). MiR-10a was significantly decreased in regions of athero-suscetibility with EC dysfunction (Fang, et al. 2010). Inhibiting miR-10a in ECs result in NF-ƙB signal pathway activation and increase of the pro-inflammatory gene expression (Fang, et al. 2010). Unsurprisingly, miR-126 was reported to promote EC regeneration and repair after injury (Schober, et al. 2014). Additionally, miR-126 deletion/inhibition resulted in a thicker neointimal lesion, and administration of miR-126 mimics could rescue the dysregulated ECs caused by hyperlipidaemia (Schober, et al. 2014). MiR-181b is another miRNA that plays an important role in modulating the NF-ƙB signal pathway and suppressing inflammatory gene expression in vivo after treatment with TNFα (Sun, et al. 2012). Interestingly, the SMC-specific miRNA, miR-145 has also been reported to play a role in ECs by targeting junctional adhesion

molecule-A (JAM-A) (Schmitt, et al. 2014). Additional study showed that shear stress could increase miR-143/145 expression in ECs and promote SMCs being switched to an athero-protective phenotype, preventing atherosclerotic lesion formation (Hergenreider, et al. 2012). These data suggest that physiological shear stress could trigger the communication between ECs and SMCs through releasing miRNAs, and consequently prevent atherosclerosis.

Dysregulated cholesterol metabolism and homeostasis is a critical factor underlying atherosclerosis development and progression. Several miRNAs have been reported to be involved in the dysregulation of cholesterol metabolism and homeostasis, and consequently atherosclerotic plaque formation. In this regard, liver-specific miRNA, miR-122 (Jopling, et al. 2005) has been identified as a key regulator to control HCV replication as well as cholesterol metabolism and homeostasis. Inhibiting the miR-122 in liver reduces plasma cholesterol level in mice (Elmen, et al. 2008). Apart from miR-122, miR-33 is another miRNA, its role in cholesterol metabolism and homeostasis, and atherosclerosis has been extensively studied. MiR-33 is located in the intron of Srebp2 gene, a gene belongs to sterol regulatory element-binding protein (SREBP) group (Rayner, et al. 2010). As expected, miR-33 decreased plasma High-density lipoprotein (HDL) level by targeting human ATP-binding cassette transporter A1 (ABCA1) (Rayner, et al. 2010). Inflammatory responses and lipid uptake inside the intima by macrophages and VSMCs induce plaque development. MiR-155 regulates the macrophage inflammatory response though modulating CCL2 gene expression and thus activating the NF-kB pathway (Nazari-Jahantigh, et al. 2012). An early study reported a role for miR-21 in controlling VSMC

proliferation and apoptosis, and promoting neointimal formation in rat carotid artery injury model (Ji, et al. 2007). MiR-26a has also been suggested to play a role in VSMC differentiation though regulating TGFβ1 signalling pathway (Leeper, et al. 2011). Both abovementioned miRNAs function as an inhibitor of SMC differentiation, but an opposite role was reported with other miRNAs including miR-145. Moreover, miR-145 was down-regulated in human atherosclerosis samples, while overexpression of miR-145 in animal models could significantly reduce the plaque size (Lovren, et al. 2012). These reports suggest that VSMC phenotype switch is finely controlled by multiple miRNAs; any change within the miRNA regulatory network could break the balance and trigger the disease.

Atherosclerotic angiogenesis (new vessel formation) is developed at the later stage of the atherosclerosis. It has been suggested that macrophages within atherosclerotic plaque could release cytokines into microenvironment, which in turn promote atherosclerotic angiogenesis. Neo-vessel formation in the plaque is a very complex process and controlled by a complicated regulatory network including many miRNAs. One of such miRNAs is miR-222. It has been reported that miR-222 regulates EC migration and proliferation through Signal Transducer and Activator of Transcription 5A (STAT5A). *In vivo* experiment further confirmed that STAT5A was the target gene of miR-222, and proven that miR-222 could reduce the neo-angiogenesis and intra-plaque vessel formation (Dentelli, et al. 2010). Neovessel formation in the plaque introduce a new challenge in atherosclerosis since the haemorrhage due to the leaky neovessels within plaque could attract and bring more inflammatory cells into plaque and produce more pro-inflammatory cytokines, which in turn increase plaque

vulnerability and cause plaque rupture. At this scenario, the pro-inflammatory cytokines and other apoptosis factors trigger EC and VSMC death which significantly aggravate the plaque instability and result in rupture. In this regard, growing evidence has suggested an important role for multiple miRNAs in cell apoptosis. Specifically, miR-365 could prevent EC from apoptosis induced by ox-LDL via targeting anti-apoptotic gene Bcl-2 (Qin, et al. 2011). A similar role was observed for miR-21 in hydrogen peroxide (H_2O_2) induced VSMC apoptosis (Lin, et al. 2009). Interestingly, miR-221/222 has a pro-proliferation effect in VSMCs (Liu, et al. 2009), but an opposite effect was observed in ECs (Dentelli, et al. 2010). MiR-221/222 expression was after angioplasty, inhibiting these two miRNAs expression in the rat carotid artery after injury could reverse neointima formation by promoting re-endothelialization of the injured arteries. These findings indicate that miRNAs regulate cellular functions in a cell-specific manner (Liu, et al. 2012).

Apart from atherosclerosis and neointima SMC hyperplasia, miRNAs have also been extensively implicated in other CVDs, such as heart failure (HF) and acute myocardial infarction (AMI). Heart failure is a final manifestation of CVD which happened when the heart cannot providing enough circulatory force to pump sufficient blood maintaining the body's need (Leonard, et al. 2012). The main pathological characteristics of heart failure is associate with the damaged cardiac tissue replaced by accumulating ECM and activating cardiac fibroblasts, resulting in cardiac fibrosis and eventually HF (Mann, et al. 2005). Extensive studies have unraveled the great importance for many miRNAs in cardio-myocyte dysfunction, cardiac fibrosis and thus HF. Decreased level of miR-133 was observed in both

murine model of cardiac hypertrophy and patients with heart diseases (e.g., hypertrophic cardiomyopathy and atrial dilatation) (Care, et al. 2007). Moreover, many dysregulated serum/plasma miRNAs have been observed in patients with symptomatic HF (Sygitowicz, et al. 2015). Specifically, it has been reported that the expression levels of circulating miR-423-5p could distinguish the HF patients from the non-HF patients, inferring a diagnostic role of this miRNA in HF (Tijsen, et al. 2010). Further study demonstrated a diagnostic and prognostic potential for another two miRNAs, miR-1254 and miR-1306, as evidenced by both miR-1254 and miR-1306 were significantly associated with the increased risk of mortality and morbidity in patients with HF (Bayes-Genis, et al. 2018).

AMI is another major cause of the high morbidity and mortality rate in patients with CVD. Significant changes were observed for the expression profiles of miRNAs after AMI. In an animal AMI model, the authors reported that the expression level of serum miR-1 peaked at 6 hours post AMI, then decreased to normal level after 3 days (Townley-Tilson, et al. 2010). In patients with left ventricular remodeling (LVR) after AMI, both miR-21 and miR-146a were significantly increased 5 days post AMI, and their expression levels were correlated with AMI traditional biomarkers (Liu, et al. 2015). MiR-208a was another miRNA with an expression level peaked at 3 hours post AMI, and associated with other AMI traditional biomarkers (Bialek, et al. 2015). Compared with miR-1 and miR-208, a more accurate predictive value for AMI was observed with miR-499 (Liu, et al. 2015), suggesting miR-499 released from the damaged cardio myocytes could serve as a potential novel biomarker in AMI patients (Liu, et al. 2015; Corsten, et al. 2010). Although many miRNAs are dysregulated in AMI, not all of them are

specifically expressed in cardiomyocytes, and can be used as AMI biomarkers.

1.9 MiR-214 biogenesis

The human miR-214 gene was first discovered by a computational procedure during the sequence comparison in invertebrates (Lim, et al. 2003). MiR-214 gene is located at the opposite strand of the Dynamin-3 gene (Dnm3) intron 14, also called Dmn3os (Loebel, et al. 2005). Apart from miR-214, miR-199 is also located at this genomic region, which contains a vertebrate –specific conserved cluster of the Dnm3 gene (Desvignes, et al. 2014). Dmn3os is a 7.9kb antisense transcript of the Dmn3 gene, although it has a potential open reading frame in the mouse, a similar start site cannot be found in human and chicken species, thus Dmn3os was considered as a non-coding RNA transcript. Further genomic knock out experiment showed that deleting Dmn3os in mice resulted in a lower level of miR-199 and undetectable level of miR-214 (Watanabe, et al. 2008). Another miRNA, miR-1320, is found to be transcripted from the opposite strand of the Dnm3os (Scott, et al. 2012). It has been reported that the transcription of miR-214 expression is regulated by Twist-1, a nuclear transcription factor (Thisse, et al. 1988; Gitelman. 1997). Twist -1 promotes miR-214/199a2 expression by binding to the E-box within Dnm3 gene intron (Lee, et al. 2009). Conversely, it has been shown that several potential NF-ƙB binding sites are located in the upstream promoter region of the miR-214/199a2, and miR-214/199a2 are negatively regulated by NF-ƙB signalling (Duan, et al. 2012). Moreover, miR-214/199a2 expression is transcriptionally regulated by ZEB1, a transcription factor structurally similar to Twist-1 and functionally binding to E-boxes (Williams, et al. 2012).

Importantly, miR-214 homologs with conserved sequence have been discovered in over 20 different species, inferring a crucial role for this miRNA in variety of biological functions and diseases (Desvignes, et al. 2014; Penna, et al. 2015)(Figure 6).

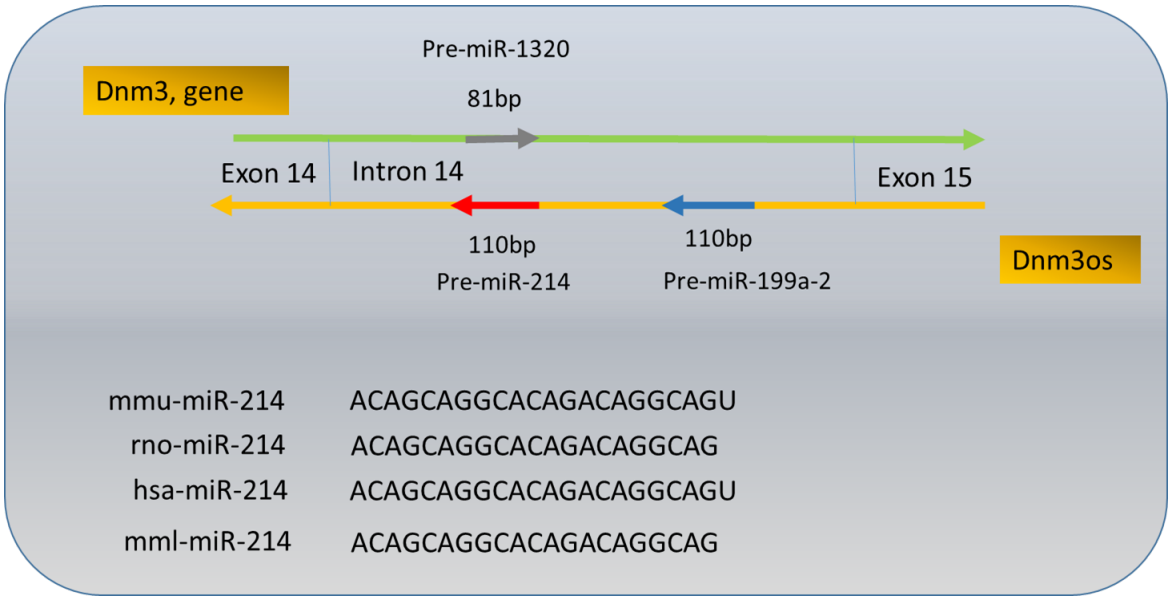


Figure 6. MiR-214 location and sequence. MiR-214 is located at the opposite strand of the Dynamin-3 gene (Dnm3) intron 14, called Dmn3os. Three different miRNAs (miR-214, miR-199, and miR-1320) are transcript from this genomic region. The sequences of mouse, rat, human and Rhesus macaque miR-214 were included here.

Indeed, spatiotemporal changes of miR-214 expression were observed in different organs and tissues during the development. MiR-214 expression in embryonic murine heart was rapidly increased at the early developmental stage and peaked at E12.5 (Aurora, et al. 2012). Interestingly, DNM3os gene inactivation leads to an abnormal development of skeletal and adipose tissues, and DNM3os mutant mice died shortly after birth (Watanabe, et al. 2008), suggesting an important role for these miRNAs during embryonic development. Indeed, zebrafish study showed that miR-214 regulates the skeletal muscle development by targeting the

Sufu gene (Flynt, et al. 2007). Additionally, it has been reported that the skeletal muscles differentiation from embryonic stem cells is regulated by miR-214, and miR-214 exerts such function via modulating Ezh2 gene expression (Juan, et al. 2009). These results suggest a role for miR-214 in the skeletal muscles differentiation. However, no embryonic lethality and abnormal phenotype is observed in global miR-214 knockout mice (Aurora, et al. 2012), inferring a redundant role for this miRNA in embryonic development. However, a pathological role was confirmed using these knockout mice, with an increased level of cardio myocytes apoptosis after AMI was determined in these mice (Aurora, et al. 2012).

1.9.1 MiR-214 in cancer

Functional importance of miR-214 has been first investigated in cancers, reported an inhibitory effect of miR-214 on cancer cell apoptosis(Cheng, et al. 2005). However, later studies point-out a contradicting role for miR-214 in different cancers. Specifically, malignant melanoma, basal cell carcinoma and squamous cell carcinoma (SCC) are three major type of skin cancers. MiR-214 is upregulate in metastatic melanoma cells and promotes melanoma metastasis by increasing the melanoma cell survival, migration and invasion (Penna, et al. 2011). On the other hand, miR-214 expression was decreased in human SCC cells (Yamane, et al. 2013), with no significant change was observed in basal cell carcinoma (Sand, et al. 2012). Moreover, miR-214 has an anti-breast cancer effect since miR-214 over-expression inhibits breast cancer cell proliferation and migration, but increases cell apoptosis (Liu, et al. 2016). Consistent with this finding, lower expression level of miR-214 was observed in breast cancer patients (Yi, et al. 2016) (Zhang, et al. 2016). However, increased level of miR-214 has been reported

in patients with ovarian cancer from two different studies (Yang, et al. 2008) (Vaksman, et al. 2011). Furthermore, miR-214 dysregulation has also been reported in patients with pancreatic cancer (Cao, et al. 2018), stomach cancer (Yang, et al. 2018), lung cancer (Zhang, et al. 2018), bladder cancer (Wang, et al. 2015), and colorectal cancers (Wu, et al. 2018). These contrasting observations highlight a divergent role for miR-214 in different cancers/diseases, with a cellular-context- and/or disease-dependent effects on cancer progression. Due to its huge importance in some cancers, miR-214 represents a promising drug target in fighting with cancers. In this regard, the therapeutic effect of miR-214 on cutaneous T-cell Lymphoma (CTCL) has been tested in a pre-clinical study (Kohnken, et al. 2019).

1.9.2 MiR-214 in other diseases

MiR-214 has also been implicated in other diseases, such as DM, kidney disease and neural disease. MiR-214 overexpression prevents diabetic nephropathy by reducing oxidative stress in diabetic kidney (Yang, et al. 2019). However, another study shows that increased expression of miR-214 in renal tubular cells induce the mitochondrial dysfunction and lead to the renal tubular cell death (Bai, et al. 2019). Additionally, decreased serum miR-214 level was observed in patients with ankylosing spondylitis. Since precise and rapid distinguishing the patients with or without ankylosing spondylitis is crucial for the patients prognosis and their quality of life, circulating miR-214 could serve as a novel biomarker for diagnosing this disease (Hyun Yi Kook, et al. 2019). Moreover, it has been reported that inhibiting miR-214 after spinal cord injury reduces oxidative stress, promotes neural cell survival and inhibits inflammatory diffusion at the injury site (Wang, et al. 2019).

1.9.3 MiR-214 in CVDs

Similar to cancers, a contradicting role for miR-214 in CVD has also been documented in literature. Increased serum miR-214 level was reported in patients with HF, and correcting the dysregulated miR-214 expression level could reverse the cardiac remodeling *in vivo* (Duan, et al. 2015). In this thesis, the authors also reported a role for miR-214 in EC function and angiogenesis (Duan, et al. 2015). A later study also showed that miR-214 expression is dramatically decreased in mice after treatment with Doxorubicin (DOX), a drug has been widely used for treating cancers with a proven side-effect, causing the congestive heart failure (Octavia, et al. 2012). Interesting, miR-214 expression in these patients displayed a time and dose dependent manner (Holmgren, et al. 2016). Above studies suggest that miR-214 could be a potential biomarker in HF.

MiR-214 seems to play a more consistent role in ischemic cardiac disease. Study showed that miR-214 plays a protective role in AMI by reducing cardiomyocyte death and activating cell proliferation signal pathway, which in turn reduces the infarct size and improve the heart function. PI3K/Akt signalling pathway is one of the most important pathways controlling multiple cellular functions such as apoptosis, cell cycle progression, proliferation, and growth (Cantley. 2002). Phosphatase and tensin homologue (PTEN) reduces the Akt phosphorylation and inhibits the PI3K/Akt signal pathway, thus increases cell death in the ischemic cardiac tissue (Gericke, et al. 2006; Stambolic, et al. 1998). In cardiomyocytes, miR-214 promotes Akt phosphorylation and activates PI3K/Akt signalling by targeting PTEN. Animal study showed that miR-214 overexpression reduced

myocardial infarct size after ischemia/reoxyenation (I/R) through promoting Bad protein phosphorylation and increasing Bcl-2 protein level (Wang, et al. 2016). Increasing in reactive oxygen species (ROS) after I/R induces the cell apoptosis. Decreased expression of miR-214 was observed in cardiomyocytes in response to oxidative stress, and miR-214 overexpression prevented ROS-induced cell death via modulating PTEN expression level. Thus, aforementioned studies have provided clear evidence to support a protective role for miR-214 after AMI. MiR-214 exerts such an effect by inhibiting PTEN and activing PI3K/Akt pathway. Apart from activing cell proliferation signal pathway, miR-214 protects cells from apoptosis by also supressing some pro-apoptosis protein expression. Ca^{2+} accumulation in the cardiomyocytes after I/R is another key underlying cause of cell death. Sodium/calcium exchanger1 (NCX1) is a Ca^{2+} transporter. Mice with miR-214 inhibition treatment had a higher percentage of cell apoptosis after I/R injury and an increased level of NCX1 protein. Further evidence confirmed that NCX1 is a target gene of miR-214 in cardiac cells (Aurora, et al. 2012). Coronary artery disease (CAD) is another common ischemic heart disease, resulting mainly from atherosclerosis. One clinical study reported a decreased level of circulating miR-214 in CAD patients, and showed that such a decline was associated with the severity of coronary artery stenosis (Lu, et al. 2013). Above studies highlight a protective role for miR-214 in ischemic cardiac disease, thus correcting the dysregulated miR-214 in these pathological conditions might provide a novel therapeutic strategy to treat ischemic cardiac diseases including AMI and CAD.

Cardiac hypertrophy precedes HF. Administration of isoproterenol (ISO) and phenylephrine (PE) receptor activators into mice are the two well-

reported animal models of cardiac hypertrophy. It has been shown that miR-214 expression was significantly increased in ISO-induced cardiac hypertrophy (Hou, et al. 2012). Similar data was reported in another study. Interestingly, the authors also reported that miR-214 could down-regulate X-box binding protein1 (XBP1) expression, a regulator of unfolded protein response involved in variety of signalling which control cell proliferation and angiogenesis in ischemic tissues and sustain the normal heart function. *In vivo* data revealed a negative relationship between miR-214 and XBP1 expression levels, with an increased miR-214 expression and decreased XBP1 level was observed in hypertrophic cardiac tissues (Duan, et al. 2015). Contrasting to above finding, another study showed that miR-214 expression was decreased in the hypertrophic cardiac tissues in both Ang-II infusion- or transverse aortic constriction (TAC)-induced cardiac hypertrophy animal models, and increasing miR-214 level in these mice could protect the mice against cardiac hypertrophy (Tang, et al. 2016). Above studies support the argument that miR-214 displays a model-dependent effect in cardiac hypertrophy, which should be more cautious when considering its clinical therapeutic effect in the future.

Less attention has been focused on the potential role of miR-214 in atherosclerosis and neointima formation. EC dysfunction is regarded as the starting point during atherosclerosis development and progression. One recent study has reported an increased expression of miR-214 in ECs isolated from mice with atherosclerosis, and found that miR-214 over-expression could inhibit ox-LDL-induced autophagy in young HuVECs (Wang, et al. 2018). Moreover, angiogenesis occurs at late stage of atherosclerosis, and is one of the main underlying causes of plaque rupture. In this regard,

an inhibitory effect of miR-214 on tube formation, EC migration and angiogenesis has also been reported (Chan, et al. 2009). Importantly, previous study showed that miR-214 could inhibit VSMC proliferation and migration, as well as prevent neointima formation in response to injury (Afzal, et al. 2016). Taken together, these studies imply a cellular-context- and disease-dependent involvement of miR-214 in CVD, and the functional importance in arterial remodelling still requires a further investigation.

2. Hypothesis and Aims of the PhD Project

2.1 Hypothesis

MiR-214 has been implicated in skeletal muscle differentiation (Liu, et al. 2010; Juan, et al. 2009), angiogenesis(van Mil, et al. 2012a; van Balkom, et al. 2013), heart protection from ischemic injury(Aurora, et al. 2012), bone formation (Wang, et al. 2013), and arterial remodelling(Afzal, et al. 2016). Some previous studies have provided clear evidence that miR-214 plays a role in cardiovascular development and disease. Recently, another reported also showed that miR-214 modulates SMC proliferation and migration by regulating NCAKP1 expression (Afzal, et al. 2016). However, little is known about its role in regulating iSMC differentiation from AdSPCs and its significance in injury-induced arterial remodelling.

Previous miRNA microarray analysis from Aurora' research shows that miR-214 is one of the most up-regulated miRNAs during SMC differentiation from ESCs. Moreover, miR-214 has been reported to be highly expressed at early murine embryonic stages (E10.5~E12.5) in the heart(Aurora, et al. 2012), in the vasculature rich adult organs/tissues(Aurora, et al. 2012; van Mil, et al. 2012), ECs, and VSMCs (van Mil, et al. 2012). Therefore, I hypothesized that miR-214 might play a critical role in iSMC differentiation from AdSPCs and neointima SMC hyperplasia during injury-induced arterial remodelling.

2.2 Aims

1) To investigate the functional involvement of miR-214 in SMC as well as iSMC differentiation from AdSPCs.

2) To identify and validate the potential target gene of miR-214 during SMC differentiation.

3) To delineate the underlying molecular mechanisms of miR-214-mediated iSMCs differentiation from AdSPCs.

4) To study whether AdSPC differentiation towards iSMCs is regulated in injury-induced arterial remodelling, and the therapeutic potential of in situ modulation of miR-214 expression in controlling neointimal lesion formation.

3. Materials and Methods

3.1 Materials

Key reagents used in the experimental work of this thesis are listed in Table 2.

Table 2. Reagents, kits and materials used in this project

Reagents	Supplier	Catalogue number
Acrylamide (30%)	National Diagnostics	EC-890
Ammonium Persulfate(APS)	Thermo Scientific	17874
Ampicillin	Sigma Aldrich	7177-48-2
Anti-CD31 antibody	Abcam	ab28364
Anti-CD45 antibody	Biolegend	103102
Anti-DDR2 antibody	Abcam	ab221812
Anti-Nestin antibody	Abcam	ab9498
Anti-GLI1 antibody	Abcam	ab49314
Anti-SMαA antibody	Abcam	ab32575
Anti-SM-MHC antibody	Abcam	ab53219
Anti-SM22α antibody	Abcam	ab14106
Anti-SOX1 antibody	Abcam	ab87775
Anti-SOX10 antibody	Abcam	ab216020
Anti-SOX17 antibody	Abcam	ab155402
Anti- α-tubulin antibody	Sigma Aldrich	T6074

ATP	Sigma Aldrich	A7699
bFGF	R&D Systems	233-FB-010
Bromophenol Blue	Sigma Aldrich	GE17-1329-01
B-27™ Supplement (50X)	Invitrogen	17504044
Bovine Serum Albumin (BSA)	Sigma Aldrich	05479
Cel-miR-67 agomir	Creative-biogene	
Cell Strainer	Sigma-Aldrich	CLS431751-50EA
CellStart	Invitrogen	A1014201
Chick embryo extract	MP Biomedical	SKU 092850145
Chloroform	Sigma Aldrich	C2432
Cignal GLI Reporter (luc) Kit	Qiagen	CCS-6030L
Collagenase, Type II	Gibco	17101015
Coelenterazine	Promega	S2001
d-luciferin	Goldbio	LUCK-100
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D4540
Dithiothreitol (DTT)	Sigma Aldrich	D9779
Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 647)	Abcam	ab150075
dNTP Mix	Thermo Fisher	R0192
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich	D6546

ECL Plus Western Blotting Substrate	Thermo Scientific	32132
EcoRI restriction enzyme	NEB	R3101S
EquiPhi29™ DNA Polymerase (PCR enzyme)	Thermo Fisher	A39390
EquiPhi29™ DNA Polymerase Reaction Buffer (10X)	Thermo Fisher	B39
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E6758
Formaldehyde	Sigma Aldrich	47608
Fluoromount-G™	Thermo Scientific	00-4958-02
Gelatin	Sigma Aldrich	G1890
GelRed Nucleic Acid Stain	Biotium	41003
GenElute Mammalian Total RNA Miniperp Kit	Sigma Aldrich	RTN350
GenElute™ Plasmid Miniprep Kit	Sigma Aldrich	PFM250
Glycerol	Sigma Aldrich	G5516
Glycine	Sigma Aldrich	50046
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488)	Abcam	ab150113
Goat Anti-Mouse IgG H&L (Alexa Fluor® 647)	Abcam	ab150115

Goat Anti-Mouse IgG H&L(HRP)	Abcam	ab97040
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	ab205718
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Abcam	ab150077
Hanks' balanced salt solution (HBSS)	Sigma Aldrich	H6648
HEPS	Sigma Aldrich	54457
IFNγ Mouse ELISA Kit	Thermo Scientific	KMC4021
IL-6 Mouse ELISA Kit	Thermo Scientific	EM2IL6
Isopropanol	Sigma Aldrich	W292907
JM109 competent	Promega	L2005
KAPA SYBR FAST qPCR Kit Master Mix	KAPA Biosystems	07959621001
KOH	Sigma Aldrich	720143
LiCl	Sigma Aldrich	L4408
L-Glutamine	Gibco	25030081
MiR-214 mimics	Sigma Aldrich	HMI0379
MiR-214 inhibitor	Sigma Aldrich	HLTUD0379
MiRNA negative control	Sigma Aldrich	HMC0002
MCP-1 Mouse ELISA Kit	Thermo Scientific	EMMCP1

$MgSO_4.7H_2O$	Sigma Aldrich	63138
MiRNA mimic,Negative control	Sigma Aldrich	HMC0002
MiRNA Inhibitor, Negative Control	Sigma Aldrich	4464079
MiR-214 Agomir	Creative-biogene	
MiR-214 inhibitor	Sigma Aldrich	HLTUD0378
MiR-214 mimic	Sigma Aldrich	HMI0379-5NMOL
Mouse IgG control	Abcam	ab37355
Murine s100a4 ELISA Kit	Antibody Online	ABIN4972603
NaCl	Sigma Aldrich	S9888
NCode VILO miRNA cDNA synthesis Kit	Invitrogen	MIRAS-20
NotI restriction enzyme	NEB	R3189S
NP-40	Thermo Scientific	85125
Paraformaldehyde (PFA)	Sigma Aldrich	P6148
pCDH-CMV-MCS-EF1-Puro vector	System Biosciences	CD510B-1
pCMV-Green Renilla Luc vector	Thermo Scientific	16153
pMCP-1 Plasmid	Addgene	#40324
pmIL-6 FL Plasmid	Addgene	#61286

Polyvinylidene difuoride (PVDF) membranes (0.45mm)	GE Healthcare	10600038
Phosphate buffered saline (PBS) 10×	Sigma Aldrich	P5493
Pierce™ BCA Protein Assay Kit	Thermo Scientific	23225
Prestained Protein Ladder	Thermo Scientific	26616
Penicillin(10,000unit/ml)-streptomycin (10mg/ml)combination (P/S) P/S	Sigma Aldrich	V900929
proteinase K (20 mg/mL)	Thermo Scientific	25530049
Protein G Magnetic Beads	Thermo Scientific	88848
Protease Inhibitors	Sigma Aldrich	S8820
PureLink™ Quick Gel Extraction Kit	Invitrogen	K210012
Puromycin	Sigma Aldrich	P9620
QuikChange Lightning site-Directed mutagenesis Kit	Agilent	210515
Rabbit IgG control	Abcam	ab172730
Recombinant Mouse TGF-beta 1 Protein (TGFβ1)	R&D Systems	7666-MB-005
Recombinant Mouse TNF-alpha Protein (TNFα)	R&D Systems	410-MT-010

Reporter Lysis 5X Buffer	Promega	E3971
Retinoic acid	Sigma Aldrich	R2625
RevertAid Reverse Transcriptase Kit	Thermo Scientific	K1691
RIPA Lysis and Extraction Buffer	Thermo Scientific	89900
RNaseA	Thermo Scientific	12091021
ShRNA Sufu plasmids	Sigma Aldrich	SHCLNG-NM_015752
ShRNA Non-Targeting control vector	Sigma Aldrich	SHC002
Sodium dodecyl sulphate (SDS)	Sigma Aldrich	L3771
Sodium Deoxycholate	Sigma Aldrich	30970
Sufu cDNA ORF clone	GeneScript	NM_001025391
TAE buffer 50×	Sigma Aldrich	1061741000
tetramethylethylenediamine (TEMED)	Thermo Scientific	17919
Tips of cross-IT 200x guide wire	Abbott Laboratories	0030281
TRI-reagent	Sigma Aldrich	93289
Tris Base	Sigma Aldrich	T1503

Tris-HCl	Sigma Aldrich	T3253
Triton X-100	Sigma Aldrich	T8787
TurboFect Transfection	Thermo Fisher	R0533
T4 DNA Ligase	Invitrogen	15224090
Tweet-20	Sigma Aldrich	P7949
2-mercaptoethanol (β-ME)	Sigma Aldrich	M3148
4',6-diamidino-2-phenylindole (DAPI)	Sigma Aldrich	D9542

3.2 AdSPC isolation

The procedures for mouse aortic AdSPC isolation and culture were followed the protocols described in Tang's study (Tang, et al. 2012), details are described below.

The following media were prepared before beginning experiments:

·GP medium: 50% P/S mixed with 50% L-Glutamine.

·Tissue collection medium: 89% DMEM mixed with 10% FBS and 1% GP medium.

·Perfusion medium: HBSS mixed with 1% GP medium.

·Gelatin medium: PBS with 0.1% Gelatin.

·P/S medium: PBS with 1% P/S.

·Digestion medium: 1mg/ml and 3mg/ml Collagenase II in DMEM.

·Elastase medium: 0.744 U/ml Elastase in DMEM.

·Operation medium: 89% DMEM mixed with 10% FBS and 1% GP medium.

.CellStart medium: PBS with 1% CellStart reagent. Put on the ice before use.

Before experiments the surgical instruments and culture dishes were irradiated with UV light for at least 30min and 12-well plates were coated with CellStart for at least 30min.

Asphyxiated mouse with CO_2 for at least 2min, sprayed the mouse with 75% ethanol thoroughly. Opened the chest carefully without cutting any vessels with sterilized forceps and scissors. Perfused the mouse through a cardiac puncture at the apex with Perfusion medium. Removed the organs if necessary to make sure there is clear enough view of the whole aorta before cut it down. Removed the connective tissue around the aorta carefully. Cut down the aorta after it is free of fibrous material and fat tissue (Approximately 1.5cm long). Placed the aorta into the collection medium and keep them on ice before use.

Rinsed the aorta in operation medium quickly and placed it in the 10cm culture dishes covering with tissue collection medium. Scraped off as much surrounding fat tissue of the artery as possible under microscope. Aortas were washed three times with PBS supplemented with 1% P/S. Digested the artery with 1 mg/ml Collagenase II in a new dish at 37°C for 15min. Carefully put the aorta into the new dish containing collection medium. Adventitia were carefully dissected away under a dissecting microscope. Unfolded and cut the adventitia layer into cubes as small as possible with scissors. Tissue blocks from 10 mice were pooled together.

Transported the cubes and the medium into a 15ml centrifuge tube, spined down the cubes at 800rpm for 3min. Incubated the tissue blocks with 3 mg/ml type II collagenase in DMEM with a 1/5 (w/v) ratio of tissue (g) to

enzyme solution (ml). After incubated for 30 min, the same volume of 1 mg/ml elastase solution was added to the solution contain the tissue and collagenase. The tissues were incubated for another 1–2hr until all the tissues were digested. Flited the cells with a Cell Strainer (70μm). Spined down the cell digestion solution at 800rpm for 3min and suspend cells in culture medium. Seeded the cells in pre-coated 12 well plates and culture at 37°C in 5% CO_2 incubator. (2-3 aortas in one well of 12-well plate)

3.3 AdSPC culture and maintenance

The following media were prepared before beginning experiments:

·AdSPC culture medium: DMEM supplemented with 2% chick embryo extract, 1% FBS, 1% N2 supplement, 2% B27 supplement, 100nM retinoic acid, 50nM β-ME, 1% P/S and 20ng/ml bFGF.

·Gelatin medium: 0.04% Gelatin in PBS.

·DMEM medium: DMEM mixed with 10% FBS.

·Freezing medium: FBS 20% mixed with 70% DMEM and 10% DMSO.

· SMC differentiation induction medium: DMEM supplemented with 5% FBS and 5ng/ml TGFβ1.

·iSMC differentiation induction medium: DMEM supplemented with 5% FBS, 5ng/ml TGFβ1 and 50ng/ml TNFα.

AdSPC passaging

AdSPCs were cultured/maintained in T25/T75 flasks with 5/15ml AdSPC culture medium at 37°C under constant supply of 5% CO_2. Cells were observed every day to check cellular growth and morphology. Cell passaging was required once cell reach confluence level of 80-90%. Used Gelatin medium to coat the flasks for 30min before experiment. Washed the cells with PBS 2-3 times, then added 0.5/1ml Trypsin-EDTA to the

T25/T75 flask accordingly, shaked the flasks gently and put flask into the incubator for 30sec to 1min. Closely inspected the cell digestion under the microscope, Added DMEM medium to stop the digestion once cells start detaching from the flasks. Collected the cells with 15ml centrifuge tube and spined down the cells at 800rpm for 3min.Suspended the cells in AdSPC culture medium and re-plateed cells into pre-coated flasks at a ratio of 1:3.

AdSPCs freezing

AdSPCs were preserved in freezing medium in -80°C freezers (for short term) or in liquid nitrogen (for long term preservation). When the cultured cells reach a confluence level of 80-90%, detached cells as described in AdSPC passage. Collected the cells with 15ml centrifuge tube and spined down the cells at 800rpm for 3min. Discarded the supernatant and re-suspended the cells by adding suitable quantity of freezing medium (usually 1 ml for each cryovials). Transferred the cells to freezing container and placed in -80°C freezers overnight. For longer preservation, the cryovials were transferred from -80°C freezers to liquid nitrogen.

AdSPC thawing and recovery

Took the cryovials containing cells from liquid nitrogen and placed the cryovial into the 37°C water bath. After thawing, transferred the cell suspension into 15ml centrifuge tube and added 5ml pre-warmed DMEM medium into the cell suspension. Centrifuged the cells at 800 ×g for 3min, discarded the supernatant and re-suspended the cells with AdSPCs culture medium. Transferred the cell suspension into T25/75 flasks pre-coated with Gelatine medium and cultured in 37°C incubator with 5% CO_2.

SMC or iSMC differentiation from AdSPCs

Undifferentiated AdSPCs (p3~p8) were used for differentiation. For SMC differentiation, incubated the cells with SMC differentiation induction medium for up to 8 days in different experiments. For iSMC differentiation, treated the cells with iSMC differentiation induction medium for 2 to 4 days. Differentiation medium was refreshed every other day.

3.4 Genetic material transfection

Washed the cells one or two times with PBS before treated with trypsin-EDTA and subsequently neutralized by adding DMEM medium. Collected the cells and centrifuge at 800 ×g for 3min, discarded the supernatant and re-suspended the cells with 5ml AdSPCs culture medium. Used the haemocytometer to count the cells, seeded $1.5x10^5$ cell per well of 6 well plate pre-coated with Gelatin medium. Cultured the cells overnight before transfection. Clean Eppendorf tubes were labelled for each treatment, added 2µg/µl TurboFect Transfection Reagent and 500µl serum free DMEM into the Eppendorf tubes. Added 5µl of miR-214 mimics/inhibitor or their respective control scramble miRNA mimics/inhibitor into the corresponding tube (for plasmid transfection, add 1µg plasmid) for each well of 6-wells plate. Mixed the mixture and left at room temperature for 20-30min to form the transfection complexes. Refreshed the cells with culture medium or treatment medium before transfection. Added the mixture drop by drop with circular motion to dispose the transfection mixture as evenly as possible. Rocked back-and-forth and side-to-side the 6-well plate immediately to evenly distribute the mixture. Incubated the cell for 24-48 hrs at 37°C in 5% CO_2 incubator for further experiments.

3.5 DNA amplification, purification, and transformation

DNA product without restriction enzymes site or not enough amount need to be amplified by Polymerase Chain Reaction (PCR) before use. Prepared the PCR reaction mixture in PCR tubes as shown in Table 3.

Table 3. PCR reaction mixture components

components	volume
10× DreamTaq Buffer	5µl
dNTP mixture	5µl (2mM)
Forward and reverse primers	0.1-1.0µM
DNA Polymerase	1.25U
DNA template	X µl (10pg-1µg)
nuclease free water	Up to 50µl
Total volume	50µl

Placed the PCR reaction mixture PCR tubes into a thermal cycler, ran the PCR program as shown in the Table 4.

Table 4. PCR reaction cycler program

step	Temperature(°C)	time	Number of cycles
Initial Denaturation	95	5min	1
Denaturation	95	1min	35
Annealing	55 (adjust according to the primer Tm)	1min	
Extension	72	1min/kb	
Final Extension	72	5-10min	1

Agarose gel was used here to detect the PCR product. 1% agarose gel was used in this study. 50ml 1xTAE mixed with 0.5g agarose, then boiled the mixture until the solution became clear, cooled the gel mixture to about 50°C. Added the GelRed Nucleic Acid Stain before poured the gel to the gel tank with a comb. Left it for about 30min to solidify the agarose gel. Transferred the gel to a gel-running reservoir, full filled the running reservoir with the 1x TAE buffer. Loaded about 20 to 30µl of PCR product each well along with the 10µl DNA ladder. Ran the gel by apply the electrical current of 120V for about 40min.Took the florescent image by using the Aphalamager HP system (Alpha Innotech, 3089N IMAGER) at dual wavelength ultra violet illumination (365nm or 302 nm) to check the PCR result.

The fragment after treatment with restriction enzymes need to be purified before use. Ran the DNA agarose gel to separate the different size DNA fragments, recycled target DNA fragments. Mixed the digested DNA fragments or recycled DNA fragment gel with 300ul Gel Solubilisation Buffer,

then pipetted the solution into a column set after the gel dissolved. Centrifuged the column at 12,000 ×g for 1min, discarded the flow through and placed the column into the wash tube. Added 500µl wash buffer to the column, centrifuged the column at 12,000×g for 1min, then discarded the flow through. Placed the column back to the wash tube, centrifuged the tube at 12,000 ×g for 1 to 2min. Placed the column onto a clean tube and added about 50µl elution buffer to the centre of the column. Centrifuged the tube at 12,000×g for 1 min and collect the purified DNA fragment.

DNA transformation was conducted following procedures. Added 1µl of plasmid DNA (or 10µl ligated DNA) into 30-50µl of JM109 competent cells in pre-cold tubes on ice. Gently mixed and incubated the mixture on ice for 30min.Then, heated the mixture at 42°C for 1min and put it back onto ice for 3-5min, left it at room temperate for 5min. Added 500µl of LB medium to the mixture and incubated at 37°C for 1hr on a floor shaker at 225rpm. After incubation, centrifuged the tube at 3,000×g for 5min and discarded the supernatant, 200µl LB broth was used to re-suspended the bacteria and spread the mixture onto the surface of the LB plate with ampicillin. Incubated the plate at 37°C overnight, transferred small number of bacteria from the colonies into a 15ml tube containing 5ml LB solution. Collected the bacteria by centrifuged the tube on following day, re-suspended the bacterial pellet with 200µl Resuspension Solution. Lysed the re-suspended cells with 200µl lysis buffer. Mixed the contents by gentle inversion until the mixture becomes clear. Added about 350ul Neutralization/ Binding Buffer into the tube inverted the tube for several times. Centrifuged the tube at 12,000×g for 10min. Inserted a binding column into a provided micro centrifuge tube. Added about 500µl column Preparation solution to the column and centrifuge at 12,000×g for 30sec, and discarded the flow-

through liquid. Added the cleared lysate to the column and centrifuged at 12,000×g for 1 min. Discarded the flow –through liquid. Added about 750µl wash buffer to the column, centrifuged the tube at 12,000×g for 1min and discarded the flow –through liquid. Centrifuged the column tube again for 1 to 2min. Placed the column onto a clean collection tube. Added 50 to 100µl elution buffer to the centre of the column. Centrifuged the column for about 1min and checked the concentration of the plasmid by NanoDrop spectrophoto meter (Thermo Scientific, D-2000).

3.6 Stable overexpression or knockdown genes in AdSPCs

Designed a pair of the pri-miR-214 primers with EcoRI/NotI restrict enzyme cleavage sites on genscript websit by following the website instroduction, enter the gene sequence on the website and then check the outcome primer sequence. https://www.genscript.com/tools/pcr-primers-designer. Approximate 688bps of genomic fragment containing mmu-miR-214 precursor (110bps) and its flanking sequence (293bps and 285bps, respectively) was amplified by PCR with specific primer set as shown in table 13. The steps were present in section 3.5. Treated the insert DNA product (pri-miR-214 or Sufu) and pCDH-CMV-MCS-EF1-Puro vector with EcoRI and NotI restriction enzymes for about 1hr at 37°C. After digestion, purified the product respectively by using the DNA gel extraction kit. Details were described in section 3.5. Mixed the vector and insert DNA with a molecular ratio of 1:4 to make a 20µl reaction mixture as indicated in the Table 5 and then kept the mixture at 4°C overnight.

Table 5. DNA ligation mixture component

Component	Blunt Ends
5×Ligase Reaction Buffer	4µl
Insert Ends	X µl (45-480fmol)
Vector Ends	X µl (15-60fmol)
T4 DNA Ligase	1 µl (1unit)
Autoclaved distilled water	To 20µl

Transformed the ligation product into JM109 competent cells, and evenly smeared the transformed bacterial onto LB plate containing ampicillin. Incubated the plate at 37°C overnight. At the following day, picked single bacterial colonies from the plate and further amplified the plasmid. The insert DNA sequences were identified by PCR and double enzymatic digestion (EcoRI/NotI), and the resultant plasmids will be verified through plasmid DNA sequencing by GCTA Gene Company.

3.7 lentivirus production and infection

All vectors were verified by DNA sequencing. Control (pCDH or Lenti-GFP), Sufu (pCDH-Sufu) or miR-214 (Lenti-miR-214) pseudoviral particles were generated using pCDH, pCDH-GFP, pCDH-Sufu, or pCDH-miR-214 and packaging plasmids, respectively. Prepared enough 293T cells before experiments. Prior to transfection, the 293T cells were cultured in T75 flask for about 18hr to 24hr.Mixed the target plasmid with the packaging plasmids (pCMV-dR8.2 and pCMV-VSV-G) at the ratio 4:3:1. Added 2µg/µl TurboFect Transfection Reagent and 500µl serum free DMEM into the Eppendorf tube, mixed gently and then add mixed plasmids into the tube. Mixed the solution gently and incubate at the room temperature for about

20min.Transfected the cells overnight and refresh the medium next day. The 293T culture medium containing the lentivirus was harvested after 48h, then filtered and aliquot in 15ml falcon tubes. Keep the lentivirus in the 4 °C before use or stored at –80°C for future use.

AdSPCs were plated a day before infection in 6-wells. One transducing unit per well of miR-214 overexpression lentivirus or control virus were added into each well of the 6-well plate, then kept the infected cells in the incubator for one day. Refreshed the cells with t AdSPCs culture medium and incubated the cells for another 24hrs before treated with AdSPCs culture medium with puromycin (5μg/ml). Survival cells were checked the gene expression and subjected to other experiments.

3.8 RNA Extraction and Analysis

Total RNA Extraction

Cleaned the working bench with 75% ethanol before collect the cells. Prepared enough cells before harvest. Lift the cells using trypsin or cells scrappers and transferred the cells into a 15ml centrifuge tube. Centrifuged the cells at 800×g for 3min and discarded the supernatant. Washed the cells once with PBS and transferred the cells into a new Eppendorf tube. Centrifuged the tube at 800×g for 3min and discarded the supernatant to obtain cell pellet. Prepared the fresh lysis solution by adding β-ME at a ratio of 1:100 to lysis buffer followed the manufacturer instruction. Then added 250μl to 500μl lysis buffer to the cell pellet. Vortexed the tube until the cells were completely dissolved, transferred the mixture to the GenElute Filtration Column placed in a 2ml collection tube.Centrifugeed at 13,200 rpm for 2min at room temperature, discarded the filtration column after centrifuge. Added equal volume of 70% ethanol into the flow-through liquid.

Mixed and transfer the mixture to a clear GenElute binding column placed in 2ml collection tube and centrifuged at 13,200rpm for 30sec. Discarded the flow –through liquid. Added 500µl wash buffer 1 to the binding column and spin the tube at 13,200rpm for 30seconds. Discarded the flow –through liquid. Added 500µl wash buffer 2 to the binding column and spin the tube at 13,200rpm for 30sec. Discarded the flow –through liquid and centrifuged the binding column again for about 2min. Placed the binding column into a clean collection tube and added about 50µl elution buffer to the binding column. Centrifuged the tube at 13,200rpm for 30sec to 1min to elute the RNA samples. Checked the RNA concentration by NanoDrop spectrophoto meter (Thermo Scientific, D-2000).

MiRNA Extraction

The total RNA including the miRNAs were purified from the cells by using the TRI-reagent and DNase I digestion. Collected the cells into a 15ml centrifuge tube and prepared cell pellet as described above. Added about 500µl TRI-reagent into the tube and pipet several times to make sure the cells were lysed completely. Left the homogenised samples at room temperature for 5min. Added 100µl chloroform into the tube and vortexed the tube for 30sec. Left the mixture at room temperature for 5min and then centrifuged the samples at 12,000×g for 10min at 4 °C. Carefully collected upper colourless supernatant into a new RNase free tube. Added 500µl isopropanol to each sample and mix it gently. Incubated the mixture at room temperature for 5min, centrifuged the samples at 12,000×g for 10 min at 4 °C. Carefully discarded the supernatant and washed the pellet with 500µl cold 75% ethanol. Centrifuged the samples at 8,000 ×g for 5min at 4°C. Repeated this step once. Carefully discarded the supernatant and air-dried the RNA pellet for about 10min. Dissolved the RNA samples with

RNase free water and mixed the sample gently. Check the RNA concentration using NanoDrop spectrophoto meter (Thermo Scientific, D-2000).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For each RT-PCR reaction, 2µg purified RNA was used to prepared the reaction mixture as list in the Table 6.

Table 6. The RT-PCR sample reaction mixture

components	Volume
Total RNA	Xµl (2µg)
Random primer	2µl
RNase free water	Up to 13µl
Total	13µl

Centrifuged the reaction mixture briefly, placed the reaction tube into the thermal cycler, incubate at 65 °C for 5min, and then cool the sample down to 4°C in order to open the GC rich secondary structure and increase the primer binding effect. Prepared the master mixture for each RT-PCR reaction as shown in Table 7 while the incubation step is running.

Table 7. The RT-PCR master reaction mixture

components	Volume
5× Reaction Buffer	4µl
dNTPs	2µl
Thermo Scientific RiboLock RNase Inhibitor	0.5µl
RevertAid Reverse Transcriptase	0.5µl
Total volume	7µl

Added the master mixture into the sample reaction mixture tube, and briefly centrifuge the tube before placed into the thermal cycler. Ran the RT programme which is 25°C, 10min for annealing, 42°C, 60min for extension, 70°C, 10min for destroying the remaining enzyme, and finally cool down the samples to 4°C.

RT-PCR for miRNAs

NCode VILO miRNA cDNA synthesis Kit was used to generate miRNA cDNA. The main difference between these two RT procedures is a synthesize poly(A) tail and linker sequence will be added onto all the miRNAs, and a common and unique reverse primer is used to generate the cDNAs for all the miRNAs from the tailed miRNA population in a single reaction.

Mixed 1µg of template RNA with 4µl of 5×Reaction Mixture and 2µl of 10×SuperScript Enzyme Mixture, then topped up the volume of mixture to 20µl by adding DEPC-treated water. Briefly spine down the mixture, then incubated the PCR mixture in a PCR thermal cycler by running following programme: 37°C for 60min for RT-PCR reaction, 95°C for 5min to terminate the reaction, then cool down the sample at 4°C.

3.9 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

If the cDNA samples were kept in the freezer, thawed the sample and kept on ice before use. SYBR Green was used as a probe here to detect the cDNA quantity. SYBR green binded to newly synthesized double-stranded DNA, emitting fluorescence in direct proportion to the number of amplicons generated. A threshold line was drawn at the RFU (relative fluorescent unit) where there is a significant increase of fluorescence above background

fluorescence. The cycle number at which the samples reached this level was referred to as the cycle threshold value (CT), thus reflecting the relative abundance of the specific mRNA transcripts. Relative gene expression or mRNA/miRNA expression level was determined by the relative ratio of target gene expression level or miRNA expression level to 18S/GAPDH (taken as house-keeping gene for normal mRNAs) or U6 RNA (taken as house-keeping gene for miRNAs), respectively. The specific steps for RT-qPCR were described below.

Prepared the RT-qPCR master mixture for normal mRNAs following the Table 8.

Table 8. Components and quantity of qPCR reaction for mRNAs

Components	Volume for 1 reaction
KAPA SYBR FAST qPCR Master Mix	5µl
Forward primer	1µl (final con 0.2mM)
Reverse Primer	1µl (final con 0.2mM)
RNase free water	1µl
Total volume	8µl

Followed Table 9 to prepare miRNA RT-qPCR master mixture.

Table 9. Components and quantity of RT-qPCR reaction for miRNAs

Components	Volume for 1 reaction
SYBR GreenER RT-qPCR Master Mix	5µl
miRNA specific Forward Primer	1µl (final con 0.2mM)
Universal Reverse Primer	1µl (final con 0.2mM)
RNase free water	1µl
Total volume	8µl

After mixed, carefully disposed 8μl of the mixture into each well of 384 wells plate or 96well plate (MicroAmp Optical). Added 2μl of cDNA sample (5ng/μl) in to each reaction well and sealed the plate with MicroAmp PCR film. Briefly centrifuged the plate and placed the plate to TaqMan Real-Time PCR System (Bio-Rad, CFX96 Dx) to run the qPCR reaction. The qPCR programme was shown in the Table 10. The results were expressed as relative units based on $2^{-\Delta\Delta CT}$ calculation (Pfaffl and Hageleit 2001) , which gives the relative amount of gene normalized to endogenous housekeeping gene.

Table 10. The RT-qPCR programmes

step	Temperature(°C)	time	Number of cycles
Initial Denaturation	95	10min	1
Denaturation Annealing	95 60	10sec 30sec	40
Melt curve cooling	60-95 4	1 min	1

3.10 Gene cloning and Mutation

Sufu 3'UTR reporter with miR-214 binding site mutation and SMC gene promoters with GLI mutants

The mutation primers were designed on the website of the company the mutation kit was used, Agilent Company, entered the gene sequence and the mutation site on the website and then selected the outcome primer sequence. All the mutation primers' sequences were listed in the table 13. (https://www.agilent.com/store/primerDesignProgram.jsp) Prepared the

mutation reaction as outlined in the Table 11. After briefly centrifuged, placed the tube onto the thermal cycling machine.

Table 11. The mutant strand synthesis reaction mixture

Reaction component	Volume
10× QuikChange Lightning Multi reaction buffer	2.5µl
QuikSolution	0-0.75µl(titrate for each templat)
ds-DNA template	X µl (100ng)
mutagenic primers	X µl (100ng each primer for 1-3 primer; 50ng each primer for 4-5 primer)
dNTP mix	1µl
QuikChange Lightning Multi enzyme blend	1µl
double-distilled H_20	X µl up to 25µl

Ran the PCR programme as indicated in the Table 12. At the end of programme, placed the tube on ice. Added 1µl of Dpn1 restriction enzyme directly to the reaction tube. Gently mixed the reaction mixture by pipetting the solution up and down. Then incubated the reaction tube at 37°C for 5min.

Table 12. The mutation PCR programme

step	Temperature(°C)	time	Number of cycles
1	95	2 min	1
2	95 55 65	20 sec 30 sec 30sec/kb of plasmid length	30
3	65	5min	1

Thawed the XL10-Gold ultracompetent cells on ice and added 2µl of the β-ME into the cells to increaseed the transformation efficiency. Transferred 1.5µl PCR product into the ultracompetent cells, mixed the transformation reaction gently, incubated the reaction on ice for 30min. Heat-pulse the tubes at 42°C for 30sec and then put the tube back on ice for 2min. After that added 0.5ml LB broth to the tube and incubate it at 37°C for 1 hr on a shaker. Spread the product on the agar plates containing the antibiotic (ampicillin). Incubated at 37°C overnight, then transferred small number of bacteria from each bacterial colony into 5ml new LB broth, and incubated it on a shaker (225-250 rpm) at 37°C for 18hrs. Extract plasmids from the bacteria as described in section 3.5, and verified DNA mutation by gene sequencing.

Table 13. Primer sets used in the present study

Gene names	Forward (5'-3')	Reverse (5'-3')	Application
U6 snoRNA (mus/hu)	GATGACACGCAAATTCGTG	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
miR-214 (mus/hu)	CAGGCACAGACAGGCAGT	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
miR-34a (mus/hu)	GGCAGTGTCTTAGCTGGTTGT	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
miR-22 (mus/hu)	AAGCTGCCAGTTGAAGAACTGT	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
18s(mus/hu)	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA	Real-time RT-PCR
Mus SMαA	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG	Real-time RT-PCR
Mus SM22α	GATATGGCAGCAGTGCAGAG	AGTTGGCTGTCTGTGAAGTC	Real-time RT-PCR
Mus CNN1	GCGTCACCTCTATGATCCCAA	CCCAGACCTGGCTCAAAGAT	Real-time RT-PCR
Mus SM-MHC	AAGCAGCCAGCATCAAGGAG	AGCTCTGCCATGTCCTCCAC	Real-time RT-PCR
Mus SRF	CCTACCAGGTGTCGGAATCTGA	TCTGGATTGTGGAGGTGGTACC	Real-time RT-PCR
Mus IFNγ	CGGCACAGTCATTGAAAGCC	TGTCACCATCCTTTTGCCAGT	Real-time RT-PCR
Mus IL-6	GTGGCTAAGGACCAAGACCA	TAACGCACTAGGTTTGCCGA	Real-time RT-PCR
Mus MCP-1/CCL2	CCCCAAGAAGGAATGGGTCC	TGCTTGAGGTGGTTGTGGAA	Real-time RT-PCR
Mus S100A4	TCCTCTCTCTTGGTCTGGTCT	GTCACCCTCTTTGCCTGAGT	Real-time RT-PCR
Mus Shh	TCTCGAGACCCAACTCCGAT	GACTTGTCTCCGATCCCCAC	Real-time RT-PCR

Mus Ptch1	CCAGCGGCTACCTACTGATG	TGCCAATCAAGGAGCAGAGG	Real-time RT-PCR
Mus GLI1	GTCTGCGTGGTAGAGGGAAC	GGAGACAGCATGGCTCACTA	Real-time RT-PCR
Mus Wnt1	AACAGTAGTGGCCGATGGTG	CCAGCTGCAGACTCTTGGAA	Real-time RT-PCR
Mus Wnt4	ATCTCTTCAGCAGGTGTGGC	TGTTGTCCGAGCATCCTGAC	Real-time RT-PCR
Mus Wnt9a	CGGGGACAACCTCAAGTACA	ATCACCTTCACACCCACGAG	Real-time RT-PCR
Mus Wisp1	CACAGATGGCTGTGAATGCTG	ACCTGTGCACACACTCCTAT	Real-time RT-PCR
Mus Sufu	ACCTGCAACAGGAGAGAGTT	GATGCTCCGGCTATCCTCTTC	Real-time RT-PCR
Hu Sufu	CCACACCTGCAAGAGAGAGT	TTGGCACTGACACCACTCAG	Real-time RT-PCR
SMαA promoter-1	CATAACGAGCTGAGCTGCCTC	CCAAACAAGGAGCAAAGACG	ChIP assay
SMαA promoter-2	GATCAGAGCAAGGGGCTATA	CTACTTACCCTGACAGCGAC	ChIP assay
SMαA adjacent	GGCAACACAGGCTGGTTAAT	AGCCCCTGTCAGGCTAGTCT	ChIP assay
SRF promoter	TCAGGCCTGTGCTTTAGCCTCG	GATGGGGGCAGGGCGGAAAG	ChIP assay
SRF adjacent	CCTGGCTGGCTTGGCACTCAC	ATCTGGCCGGACGGTGTGATA	ChIP assay
pGL3-SMαA-GLI1^{mut-1}	GAGACCCAGCCTCT GGTTATTTA GATTAGAGAGTTTTGTGC	GCACAAAACTCTCTAATCTA AATAACCAGAGGCTGGGTCTC	GLI1 binding site-1 mutation
pGL3-SMαA-GLI1^{mut-2}	CAGCCTCCCGGGACATTATTTA CCCAGAGTGGAGAAGCC	GGCTTCTCCACTTGGGTAA ATAATGTCCCGGGAGGCTG	GLI1 binding site-2 Mutation

pGL3-SRF-CLI1^{mut}	GTGCTTTAGCCTCGAGATTATT TC CCACCCCCCTTTTAGCAC	GTGCTAAAAGGGGGGTGG GAAATAATCTCGAGGCTAA AGCAC	GLI1 binding site Mutation
pmiR-Luc-EVI1-WT	CTCCTC GAGCTC TCAATTTGTCTGGCCTGGGG	GAGGAG AAGCTT GTAGCCCCAGGAACTGAGT C	Mus Sufu 3'UTR reporter clone
pmiR-Luc-EVI1-BS^{mu}	CTCAGGAATGTCTAAACTCAC GCGTGGACCAGACCCTGTC	GACAGGGTCTGGTCCACGC GTGAGTTTAGACATTCCTG AG	miR-214 binding site mutation

3.11 Luciferase Assay

For Shh-GLI1 signalling reporter assay, AdSPCs were transfected with GLI and (0.15µg/2.5 x 10^4 cells) pRenilla (15ng/2.5 x 10^4 cells) mixed reporter plasmids were used. For determining the respective SMC gene promoter activity, the individual SMC gene promoter vectors (pGL3-SMαA-WT, pGL3-SM22α-WT, pGL3-SMαA-SRFmut, pGL3-SM22α-SRF^{mut}, pGL3-SMαA-$GLI1^{mut\text{-}1,\ -2,\ or\ -1/2}$, pGL3-SRF-WT, or pGL3-SRF-$GLI1^{mut}$) (0.15µg/2.5 x 10^4 cells) and pRenilla (15ng/2.5 x 10^4 cells) mixed reporter plasmids were used. Wild type plasmids were gifts form Xu's group (Margariti et al. 2009). For mouse IL-6 and MCP-1 gene promoter activity, pmIL-6 or pMCP- (0.25µg/2.5 x 10^4 cells) and pRenilla (15 ng/2.5 x 10^4 cells) mixed reporter plasmids were used. Dual-Luciferase Reporter Assay System was used for detecting luciferase and Renilla (15ng/well) activities, the details were described below.

The cells used for transfection were refreshed with the culture medium before adding the plasmid. For luciferase assay, the pRenilla vector was transfected together with the report vector or promoter vector into the cells. After 48hrs, washed the cells with PBS for one or two times, then added 100µl 1×reporter lysis buffer to each well and placed on 2D rocker for 30min at room temperature. The plate was then incubated in -80°C freezer for at least 2hs followed by thawing on a shaker at room temperature for 30min. Transferred the lysis product to 1.5ml Eppendorf tube and centrifuged at 13,200 rpm for 10min at 4°C. Prepared the Substrate buffer solution: 120mM NaCl and 25mM Tris-HCl mixed in the water then fix the pH to7.5.Prepare the luciferase reagent and Renilla substrate as outlined in the Table 14 and Table 15 below.

Table 14. The components for Renilla detection

Renilla substrate component	Volume
Substrate solution	10ml
Coelenterazine(2500ng/ul)	10µl
Total volume	~10ml

Table 15. The components for luciferase detection

luciferase reagent component	Volume
H_2O	8.7 ml
D-luc (100mM)	100µl
$MgSO_4$	100µl
ATP	100µl
HEPS(300Mm,Ph=8.0-9.0)	1ml
Total	10ml

Mixed 20µl of the sample supernatant with 100µl of the luciferase or Renilla substrate and immediately detect the activity by using a GloMax® 20/20 Luminometer (Promega, E5311).

3.12 Protein Extraction and Analysis

Prepare buffers before the experiment, 5× SDS Loading Buffer: Tris-Cl (0.25M, pH 6.8), SDS (10%), Glycerol (50%), Bromophenol Blue (0.25%), DTT (0.5 M). 10× Tris-Glycine Buffer(pH 8.4): Tris Base (30.3g), Glycine (144.1g), add water up to 1L. 1× Running Buffer: 10× Tris-Glycine Buffer(100ml), 10% SDS (10ml), add water up to 1L. 1× Transfer Buffer: 10 × Tris-Glycine Buffer(100ml), methanol (200ml), add water up to 1L. 10× TBS Buffer: Tris-

HCl(24g), Tris Base (5.6g), NaCl (88g), add water up to 1L. TBST: 10 × TBS (100 ml), Tweet-20 (1ml), Distilled water up to 1L.

Protein Extraction

Collected cells by using the Trypsin or scrapper and then centrifuged cells at 800 rpm for 5min. Discarded the supernatant and mix the cell pellet with 100-150µl RIPA Lysis and Extraction Buffer. Placed the samples on ice for about 30min, and then sonicated the samples at 4°C for 20sec to disrupt cell membrane and facilitate release of protein content. Incubated the lysate on ice for another hour while vortexed the lysate every 15min. Then centrifuged product at 13,200 rpm for 10min at 4°C. Transferred the supernatant to a new tube, measured and adjusted the protein concentration by using Pierce™ BCA Protein Assay Kit. Prepared BSA standard as shown in the Table 16 below.

Table 16. The BSA standard diluted method

Vial	Volume of diluent(µl)	Volume and source of BSA(µl)	Final BSA concentration (µg/ml)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0=Blank

Prepared the BCA working Reagent by mix the BCA Reagent A with Reagent B at ratio of 50:1. Pipetted 25µl of sample or standard into a microplate well then added 200ul reagent mixture into each well. Covered the plate and incubate at 37 °C for 30min, measured the absorbance at 562nm on a plate reader. Prepared a standard curve by plotting the BSA standard data, used this standard curve to determine the protein concentration of the sample.

Western blot

Western blot is a widely used technique in determining the protein expression in cells and tissues. Followed the Table 17 and Table 18 to prepare 10% separating and 6% stacking gels first before starting the western blot.

Table 17. Reagents for 10% separate gel

Reagents	Volume
Double distilled water	1.9ml
Acrylamide	1.7ml
Tris-HCl Buffer 1.5M (pH 8.8)	1.3ml
SDS (10%)	50µl
APS (10%)	50µl
TEMED	4µl
Total volume	~5ml

Table 18. Reagent for 5% stacking gel

Reagents	Volume
Double distilled water	2.1ml
Acrylamide	0.5ml
Tris-HCL Buffer 1.5M (pH 6.8)	0.4ml
SDS (10%)	30µl
APS (10%)	30µl
TEMED	3µl
Total volume	~3ml

Left the gel at room temperature for at least 30 min to solidify the gel eventually, remove the comb and then transferred the gel into the gel running system chamber. Added 1× running buffer to the chamber to the extent that it covers the gel and the sample loading wells. Loaded 10µl Prestained Protein Ladder into the first wells, then loaded the protein samples (the volume depends on the protein amount and the expression of the protein in cells or tissues, usually 20µg to 30µg). 80V voltage was first applied for about 20 to 30min to stacking the protein, then followed by 120V voltage for about 70-90min, until the protein marker with the smallest size reached the bottom of gel.

Transferred the protein from gel to membrane by using PVDF membrane. Activated PVDF membrane in methanol for about 5 to 10sec before using, kept the membrane in pre-cold 1× transfer buffer. After completed the gel electrophoresis, transferred the gel onto a pre- washed filter papers and then covered the gel with labelled PVDF membrane, removed the air bubble. Put the sample into the transfer cassette in correct direction. Filled the chamber with 1× transfer buffer. Electro-transferred the protein to

membrane for 2 to 3hrs at 100V, kept the transfer chamber at cool temperature (on ice). After that removed the membrane from the cassette and blocked the membrane with 10% milk diluted in 1× TBST buffer on a 2D rocker at room temperature for about 1hr.

Cut the membrane according to the marker guide and incubated the membrane with specific antibody prepared in 5% skimmed milk or BSA in 1× TBST buffer at 4°C overnight. Washed the membrane with 1× TBST buffer for 3 times before incubate the membrane with corresponding second antibody at room temperature for 1 hour. Washed the membrane with 1× TBST buffer for 3 times before incubated with ECL Plus Western Blotting Substrate for 2-3 min at room temperature. Placed the membrane into the ChemiDoc™ Touch Imaging System (Bio RAD, 12003154) to check the protein bands.

3.13 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as previously described (Yang, Chen, et al. 2019). The details were described below. Prepared buffers followed before the experiment. ChIP lysis buffer: HEPES-KOH (50mM, pH7.5), NaCl (140mM), EDTA (1mM, pH8), Triton X-100 (1%), Sodium Deoxycholate (0.1%), SDS (0.1%), Protease Inhibitors. Low salt wash buffer: SDS (0.1%), Triton X-100(1%), EDTA (2mM), Tris-HCl (20mM, pH 8.0), NaCl (150mM). High salt wash buffer: SDS (0.1%), Triton X-100(1%), EDTA (2mM), Tris-HCl (20mM, pH 8.0), NaCl (500mM). LiCl wash buffer: LiCl (0.25M), NP-40(1%), Sodium Deoxycholate (1%), EDTA (1mM), Tris-HCl (10mM, pH 8.0).

Prepared enough cells, at least two confluent 150cm^2 dishes (1×10^7-5×10^7 cells per dish) before use. Added formaldehyde directly into the dishes to

cross link the protein to the DNA, the final concentration of formaldehyde in the dishes is 0.75%. Incubated and rotated gently at room temperature for about 10min.Added glycine into the dish to stop the formaldehyde cross-linking. The final concentration of glycine in the dish is about 125 mM. Incubated and rotate gently at room temperature for about 5 min. Washed the cells for two times with 10ml cold PBS. Added 3ml PBS and scrape dishes thoroughly with a cells scraper, transferred the cells into 15ml tube. Added another 3ml PBS to dishes, and repeat the steps. Centrifuged the cells for 5min at 4°C, 1,000×g. Discarded the supernatant and re-suspend the cells in ChIP lysis buffer (750µl per dish) and then incubated the mixture on ice for 10 min. Sonicated the lysate for several times to obtain DNA fragments with sizes between 200bp and 1000bp. Different cells require different sonication times, for AdSPCs, sonicate the cells for about 38 cycles (each cycle: sonicate for 10seconds, cool-down on ice for 30 sec to 1min, with the sonication power set about 30%). After sonication, centrifuged the tubes at 4°C, 8,000×g. Transferred the supernatant to a new tube. Removed 50µl of each sonicated sample to check the DNA concentration and fragment size. Added 70ul elution buffer to 50µl sample, and then added 4.8µl of 5M NaCl and 1µl RNaseA. Incubated the mixture at 65°C while shaking overnight. Added 2ul proteinase K into the mixture and then incubated at 60°C while shaking for 1 hr. Purified the DNA, then check the DNA concentration and fragment size.

Use about 25µg of DNA per each reaction. Diluted each sample with 1:10 RIPA Buffer. For each group prepare one sample for specific antibody and another one for IgG control. Kept 50µl of sample as input. Added primary antibody or IgG control into each sample (1-10µg antibody per 25µg sample) and rotate at 4°C for 1hr. Prepared the protein G beads and add 60µl to all

samples. Rotation at 4°C overnight. Centrifuged the samples at 2,000×g for 1min and discarded the supernatant. Washed the sample once in low salt wash buffer, once in high salt wash buffer and once in LiCl wash buffer. Centrifuged the samples at 2,000×g for 1min and discarded the supernatant after each wash. Added about 120µl elution buffer to the samples and incubate the sample at 30°C for 15min on a shaker. Centrifuged the tube at 2,000×g for 1min and transferred the supernatant to a fresh tube. Added 4.8µl of 5M NaCl and 1µl RNaseA. Incubated the mixture at 65°C on a shaker overnight. Added 2µl proteinase K into the mixture and then incubated at 60°C while shaking for 1hr. Purified the DNA, then check the DNA concentration. Relative DNA levels were measured by real-time quantitative PCR.

3.14 Immunohistochemcial staining

Immunocytochemistry for cells

For immunofluorescent staining, seeded and culture 5,000-10,000 AdSPCs into each well of chamber slide until the cells reach 60-70% confluence. Discarded the culture medium and washed the chamber slide with PBS for 2 times. Then added 4% PFA to fix the cells at room temperature for about 10min. Washed the cells with PBS for 3 times, each time incubated at room temperature for 10min. Then, added 0.1% Triton x100 in PBS and incubateed the cells at room temperature for about 10 min, washed the cells for three times with PBS, 10min each time. Blocked the cells with 5% BSA in PBS at room temperature for 1hr. Incubated the cells with primary antibody or IgG control at dilution of 1 to 200 in 5% BSA with PBS medium at 4°C overnight. Next day, washed the chamber with PBS for 3 times, each for 10min. Incubated the cells with secondary antibody conjugated with fluorescence at dilution of 1 to 1000 in 5% BSA with PBS medium at room

temperature for 1hr in the closed staining container to avoid the light. Washed the cells with PBST for 3 times, each time for 10 min. Counterstained the cells with DAPI for 10min. Then, washed the cells with PBS for 3 times, each for 10 min. Removed the frame of chamber, and the slide was mounted with fluoromount-G. Took the images using the EVOS images system (ThermoFisher scientific, AMC1000).

Immunohistochemcial Staining for Sections

Frozen slide and Paraffin embedded section of mouse aorta have a little different before blocking. For the paraffin sections, the paraffin covered the sample need to be removed before staining. Incubated the paraffin section at 60°C for about half an hour, then incubated the sections in xylene for 5 min to dissolve the paraffin, transferred the sections into 100% ethanol for about 5min. After that immersed the slides in to another 100% ethanol for about 5min, then transferred the slide into 90% ethanol for about 5min. Immersed the sections into 70% ethanol and 50% ethanol for 5min in turn, transferred the slides into water for hydration. After hydration, immersed the samples into sodium citrate buffer, then placed them into the 100°C water bath for about 20min. Cooled the samples to room temperature and then washed the slide with PBS for 3 times, each for about 5min before the next step. For the frozen slide, put the slide in room temperature for about 20min before next step.

Added 4% PFA to fix the slides at room temperature for about 10min. After fixation, the slides were washed with PBS for 3 times, 10min for each time. Then incubated the slides with 0.1% Triton x100 in PBS for permeablization. 10min later, washed the slides for 3 times with PBS, 10min each time. Blocked the slides with 5% BSA in PBS for 1 hr at room temperature.

Meanwhile, prepared the primary antibody with 5% BSA in PBS. After blocking, added the primary antibody onto the tissue sections in drop wise and incubated the slides at 4°C, overnight. Washed the slides with PBS for 3 times, each for about 5 min. Incubated the slides with appropriate secondary antibody for about 1 hr in a closed container to avoid the light. Washed the slides with PBS for 3 times, each for about 5min. Then counterstained the slides with DAPI for 10min. After last wash, the slides were mounted with fluoromount-G. Take the images using the EVOS images system.

3.15 Mouse Femoral Artery Denudation Injury and miR-214 Agomir Perivascular Delivery

Animal experiments, anaesthesia and euthanasia

All animal experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). All the animal procedures were approved by Queen Mary University of London ethics review board (PPL number: PB508B78D), and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines (Guide for the care and use of laboratory animals). For mouse carotid artery denudation injury and AdSPCs transplantation, anaesthesia was induced using 100% O_2/4% isoflurane, and was maintained throughout the procedure by the administration of 100% O_2/2% isoflurane. At the end of protocol, all mice were euthanized by placing them under deep anaesthesia with 100% O_2/5% isoflurane, followed by decapitation.

Main Animal Surgical Procedures

Eight-week old C57BL/6 male mice purchased from Charles River Laboratories. Fully explored the femoral artery with a clear view, made a small incision on the artery, and carefully inserted a 0.25 mm angioplasty spring-wire into the artery through the incision. Removal of the endothelium of the femoral artery was achieved by 3~5 passages of the wire. To investigate the potential function of miR-214 function during the neointima formation, the injured femoral arteries were randomly received miR-214 or Cel-miR-67 (Negative control) agomir treatment locally. In Brief, after injury, applied about 100µl of 30% pluronic gel containing chemically modified and cholesterol conjugated 2.5nmol miR-214 or Cel-miR-67 agomirs perivascularly to the injured femoral arteries.

For AdSPC transplantation, after injury, 100µl Matrigel mixed with 20µl of cell culture medium (vehicle control), or $1x10^6$ AdSPCs infected with Lenti-GFP or Lenti-miR-214 was applied perivascularly onto the injured arteries immediately. Then the wound was closed, and the mice were scarified at day 3, day 7, day 14 and day 28 to collected the injured arteries for gene expression, immunofluorescence staining, and morphometric analysis, respectively. For neointima cell isolation. After removed the adventitial layer and the outer portion of the media under the microscope, then cut the aorta open in longitude direction and carefully remove the endothelium with a cotton swab. After that, cut the aorta into cubes as small as possible. Digested the cubes in 1mg/ml collagenase I for 3- 3.5hs, then stopped the digestion by add the culture medium. Collected the digested cells and spin down at 800rpm for 3min, and re-suspended the cells in culture DMEM medium. Seeded the cells into 12 wells plate pre-coated with Gelatine medium, and cultured the cells at 37°C in 5% CO2 incubator for 3 days before collection.

Morphometric Analysis and Quantification of Lesion Formation

The procedures used here were similar to the protocols described in Chen's study (Chen, et al. 2015). The injured femoral arteries were harvested and embedded into the paraffin. Cut and collected the sections (5um) at 100um intervals, 10 section per segment. Five digitised sections with same identification number from five segments/intervals (~0.5mm, 1.5mm, 2.5mm, 3.5mm and 4.5mm from injury site) of each animal (e.g. I-1/2, III-1/2, V-1/2 represent the 1st and 2nd section of the 1st, 3rd and 5th segment/interval, respectively) were stained with H&E for morphometric analysis.

Deparaffinize and hydrate the tissue sections as described previously. After deparaffinization and hydration, washed the slides for three times with PBS, each time for 5min. Added the Hematoxylin staining solution onto the tissue sections and incubate at room temperature for 10min. Washed out the staining solution with running water, then added 1% hydrochloric acid to remove the extra staining solution from the samples. Rinsed the slides with running water. Added alkaline blueing solution. Rinsed the slides with running water. Added Eosin Y staining solution on the top of the sample and keep it for 3min at room temperature. After staining, placed the slides into 95% and 100% ethanol, respectively for 2min each. Clear the slides with xylene twice, each time for 4min, then mounted the slide with H&E Mounting Media. Used a computerized image analysis system (pixel2, Axiovision software) to measure the external elastic membrane (EEL), internal elastic membrane (IEL), lumen, media and neointimal areas. At least ten sections from each animal were analysed and compared.

3.16 ELISA analysis of S100A4 and other cytokines

Standard preparation. Reconstituted the standard for IL-6, IFNγ, MCP-1 and S100A4 by add distilled water. Mixed the original standard tube before use. Prepared seven different tubes for different standard point, S1-7. Pipetted 225ul of distilled water into each tube, pipetted 225ul of reconstitute standard (4ng/mg) into first tube S1 and mix. Pipetted 225ul of dilution from S1 to second tube S2 and mix before the next transfer. Repeated serial dilution five more times and create S3-S7. Use distilled water as blank S0. Took out the microwell strip from the foil bag and washed each well twice with 400 µl Wash buffer. Take care of the microwell surface. After the wash, taped the microwell strips on the paper towel to dry the microwell strips before use. Immediately added 100µl of Standard dilution prepared before to the standard wells after the microwell strips were ready. Then added 50µl Sample Diluent to the Sample and blank wells. After that added 50µl of each sample to the sample wells, and 50µl Sample Diluent into the blank well, respectively. Each standard dilution and sample need at least one repeat well. Prepared Biotin- Conjugate and Streptavidin-HRP by making a 1:100 dilution of concentrated Biotin- Conjugate solution or Streptavidin-HRP Solution with Assay Buffer in a clean tube. Make sure they are used in 30 mins. Added 50ul of Biotin- Conjugate to all wells. Covered the wells with adhesive film and incubate at room temperature for about two hours on the shaker. Removed the film and washed the microwell strips for 5 times. Pipetted 100 µl diluted Streptavidin-HRP to all wells immediately after wash and cover the microwell strip with film; incubate at room temperature for one hour with shaking. Washed the strips five times. Added 100ul TMB Substrate Solution into all wells, avoid the direct exposure to intense light when incubated the wells for 30min at room temperature. Stopped the enzyme reaction by quickly added the 100µl Stop Solution into each well.

Read the absorbance of each well on spectro-photometer using 450nm as the primary wave length (optionally 620 nm as the reference wave length). Blank wells were used to adjust the plate reader before determining the absorbance of the standards and the samples.

3.17 Human Healthy and Diseased Femoral Arteries Collection

Samples of human healthy and diseased femoral arterial were collected from patients at the First Affiliated Hospital of Zhejiang University (China) from June 2013 to August 2017. All these patients were aged from 30 to 80 years who experienced elective major lower extremity amputation of the index leg. Healthy femoral arterial specimens were obtained from patients with conditions unrelated to peripheral artery disease such as trauma while diseased femoral arterial specimens were obtained from patients with peripheral artery disease, which present SMC-rich atherosclerotic lesions (identified by hematoxylin and eosin staining). Age- and sex- match were required when selected for healthy and diseased groups. Exclusion criteria was determined prior to sample collection. For instance, liver failure, dialysis because of renal failure, cancer, chemotherapy, and pregnancy, and the lack of consent to participate to the study were included in the exclusion criteria. All patients were informed and consent by written before sample collection. All procedures had local ethical approval and were approved by the Research Ethics Committees of the First Affiliated Hospital of Zhejiang University (institutional review board approval No. 2013/150). All experiments conducted in this PhD thesis followed the principles expressed in the Declaration of Helsinki. Patient feature, including demographics, comorbidities, and medical treatments, were described in Table 19.

Table 19. Baseline Patient Characteristics

Patient Characteristic	Healthy femoral artery group (HFA) (n=30)	Diseased femoral group (DFA) (n=30)	P value
Demographics			
Female sex, No. (%)	7 (23.3)	8 (26.7)	0.776
Age (years)	55.83 ± 14.05	61.97 ± 14.65	0.103
Clinical Parameters			
Current smoker, No. (%)	5 (16.67)	9 (30)	0.361
Former smoker, No. (%)	1 (3.33)	8 (26.67)	0.026*
Body mass index, kg/m2	23.04 ± 3.51	21.76 ± 3.62	0.201
Systolic blood pressure, mmHg	137.9 ± 25.53	122.23 ± 19.11	0.009**
Diastolic blood pressure, mmHg	75.87 ± 12.85	74.77 ± 13.05	0.743
Heart rate, beats /min	88.67 ± 15.66	84.6 ± 15.05	0.309
Comorbidities			
Prior stroke, No. (%)	1 (3.33)	4 (13.33)	0.353
Carotid artery disease, No. (%)	2 (6.67)	2 (6.67)	1
Coronary artery disease, No. (%)	1 (3.33)	2 (6.67)	1

Hypertension, No. (%)	6 (20)	21 (70)	0.0001***
Type II diabetes mellitus, No. (%)	0 (0)	12 (40)	NA
Hyperlipidaemia, No. (%)	4 (13.33)	22 (73.33)	<0.0001***
Chronic kidney disease, No. (%)	0 (0)	8 (26.67)	NA
Medications			
Aspirin, No. (%)	1 (3.33)	25 (83.33)	<0.0001***
Statin, No. (%)	0 (0)	21 (70)	NA
ACEI/ARB, No. (%)	3 (10)	9 (30)	0.104
Clopidogrel, No. (%)	0 (0)	6 (20)	NA
Beta blockers, No. (%)	0 (0)	6 (20)	NA
Cilostazol, No. (%)	0 (0)	9 (30)	NA
Other antihypertensive medications, No. (%)	3 (10)	17 (56.67)	0.0003***
Other antithrombotic medications, No. (%)	2 (6.67)	28 (93.33)	<0.0001***

P value present in the table is calculated with t-test, Mann-Whitney U Test or Fisher's exact test to compare continuous variables (presented with mean ± SD) or categorical variables (presented with No. (%)), respectively. NA, not applicable. *<0.05, **<0.01, ***<0.001

3.18 Statistical Analysis

At least three independent experiments were performed for each experiment category (both *in vitro* and *in vivo*). Results were expressed as

Mean ± SEM. Statistical analysis was performed using Graphpad Prism5. Shapiro-Wilk Normality Test was used for checking the normality of the data. Two tailed unpaired student's t-test was used for comparisons between 2 groups, or one-way analysis of variance with a post hoc test of LSD was applied when more than two groups were compared if the data display a normal distribution. Conversely, non-parametric Mann-Whitney U Test or Kruskal–Wallis H test was applied for comparing two groups and three or more groups, respectively, if the data did not display normal distribution or if the number of observations from each group was smaller than 5 ($n<5$). Spearman's rank correlation analyses were conducted to characterize the relationships between the gene expression levels of miR-214 and Sufu in human arterial specimens. Alpha=0.05 was chosen as the significance level, and a value of $P < 0.05$ was considered as statistically significant.

4. Result

4.1 Important role for miR-214 in SMC differentiation from AdSPCs

AdSPCs were isolated and passaged as described in the method section. Passage 3 AdSPCs were subjected to immunofluorescence staining with antibody against Sox1, Sox10, Sox17 and Nestin, typical markers for AdSPCs, as well as some mature cells markers (CD45, DDR2, SM-MHC and CD31) (Figure 7). Strong green fluorescent signal was observed in antibody against the Sox1, Sox10, Sox17 and Nestin in the AdSPCs, while other markers are rarely negative. Indicating that these cells were positive for the typical AdSPC markers.

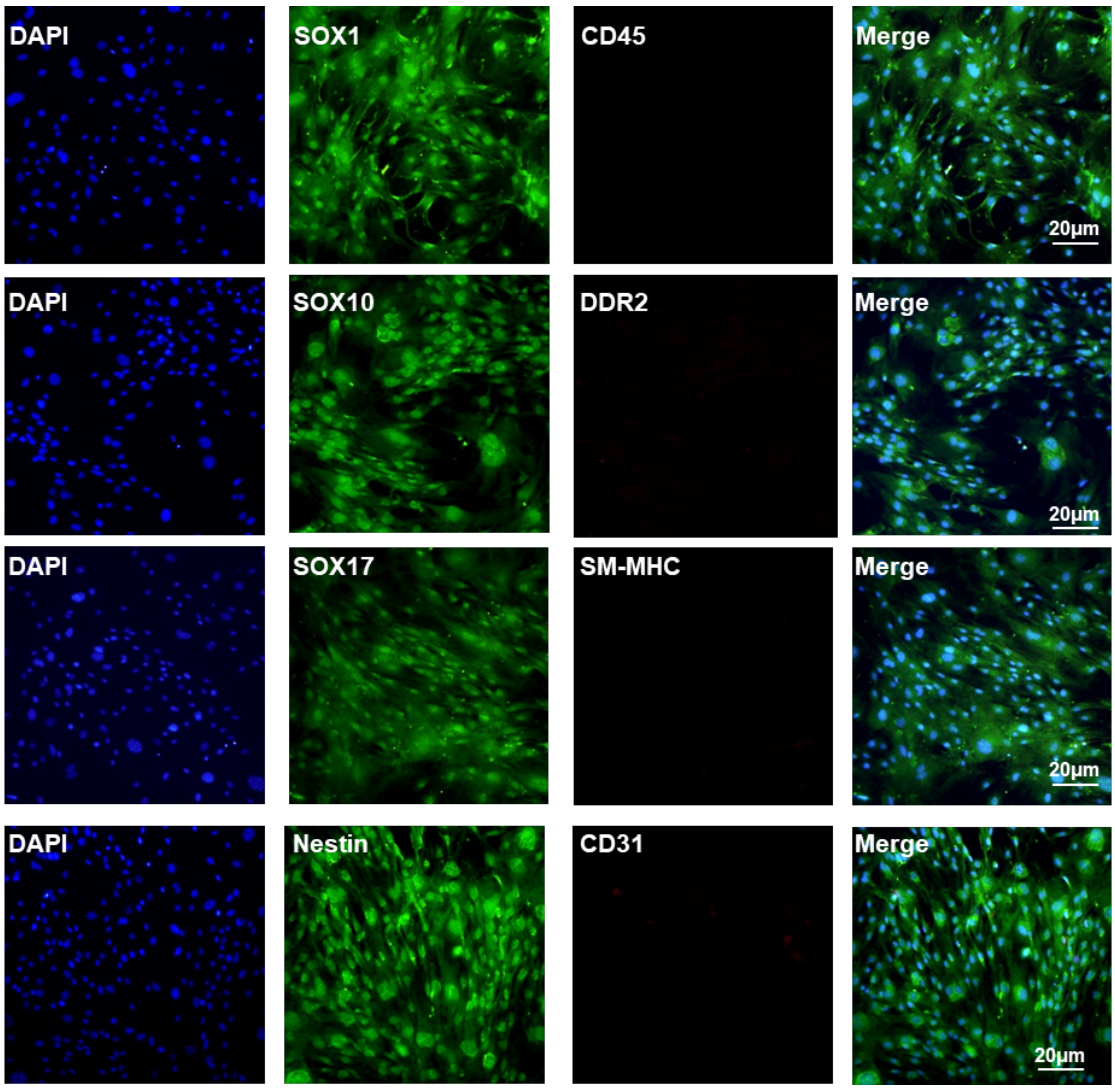


Figure 7. Immunofluorescence staining with AdSPCs. AdSPCs at passage 3 were subjected to immunofluorescence staining with antibody against Sox1, Sox10, Sox17 and Nestin, as well as some mature cells markers like CD45, DDR2, SM-MHC and CD31, respectively. Images presented here are representative of three independent experiments (n=3).

To figure out whether AdSPCs could differentiated into SMCs; AdSPCs were treated in SMC differentiation medium for day0, day2, day4, day6 and day8. Total RNAs and proteins from each group were harvested and subjected to RT-qPCR and Western blot. Some cells treated for 8 days were also subjected to immunofluorescence staining with antibody against SMαA and SM-MHC, respectively (Figure 8). Results shows that SMC markers were upregulated by cultured the AdSPCs with SMCs differentiation medium, indicate that cells were successfully induced to differentiate towards SMCs in response to SMC differentiation medium.

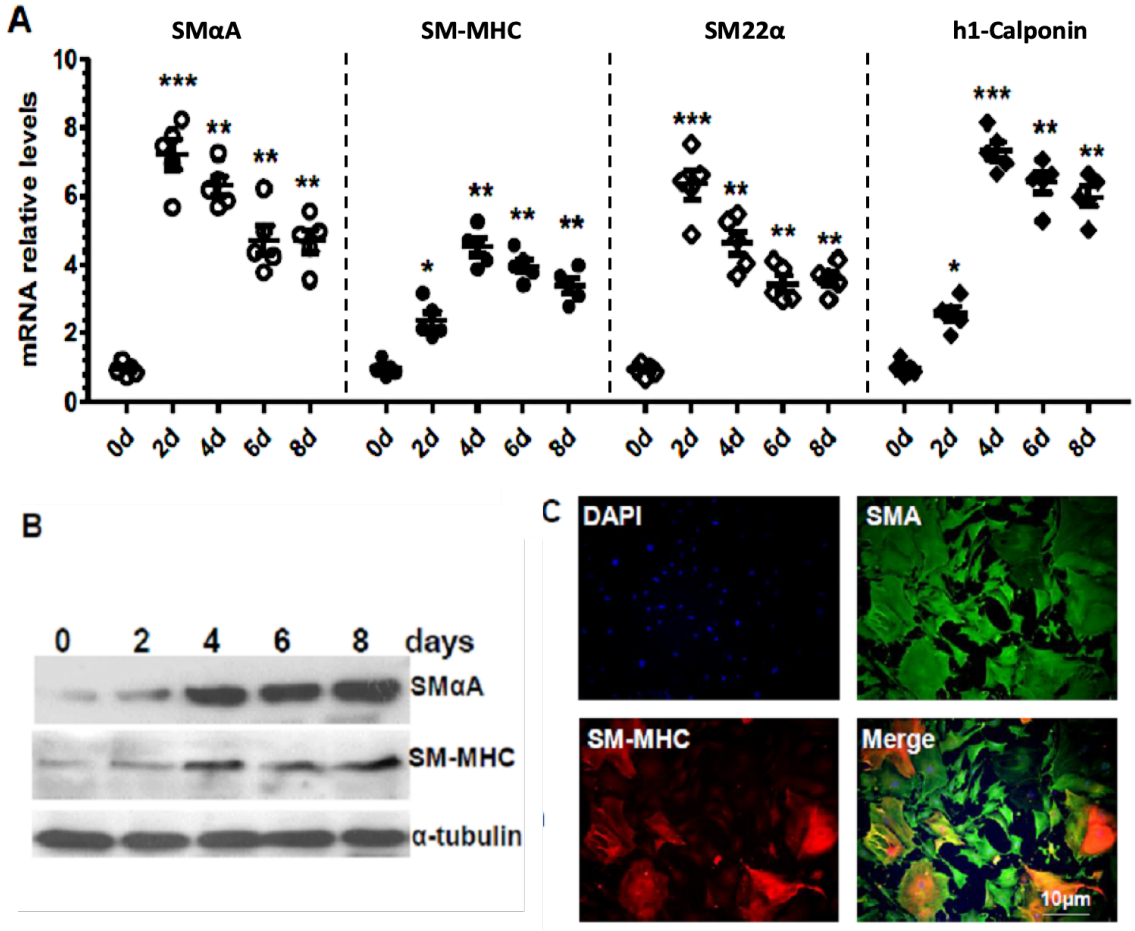


Figure 8. SMCs gene expression of undifferentiated AdSPCs and AdSPCs at day 2, 4, 6, 8.Total RNAs and proteins harvested from day 0(undifferentiated AdSPCs) and day 2, 4, 6, 8 were subjected to RT-qPCR (A) and Western blot (B) analyses, respectively. (C)Day 8 cells were stained with antibodies against SMαA and SM-MHC. The data presented here are representative images or mean±S.E.M. of five independent experiments (n=5). *P<0.05, **<0.01, ***<0.001 (versus 0d).

To study the potential involvement of miRNAs in SMC differentiation from AdSPCs, I first detected if the expression levels of miR-22, -34a and -214, the three top modulated miRNAs during SMC differentiation from embryonic stem cells (Yu, et al. 2015), were altered in SMCs differentiation from the AdSPCs. The result shows that miR-214 is the most up-regulated miRNA among them in this SMC differentiation model (Figure 9A). To study if miR-214 could play a role in SMC differentiation from AdSPCs, miR-214 gain/loss-of-function experiments were conducted in AdSPCs using either miR-214 mimics (for miR-214 over-expression) or miR-214 inhibitor (for miR-214 knockdown), respectively. As expected, miR-214 expression in AdSPCs was significantly increased by miR-214 mimics, but was decreased by miR-214 inhibitor (Figure 9B). Importantly, RT-qPCR data showed that the expression levels of all four SMC genes (SMαA, SM22α, h1-Calponin and SM-MHC) examined here were significantly up-regulated by miR-214 mimics, but down-regulated by miR-214 inhibitor (Figure 9C). Such regulation was confirmed at protein level by Western blot assays (Figure 1D & E), supporting a role for miR-214 in regulating SMC differentiation from AdSPCs.

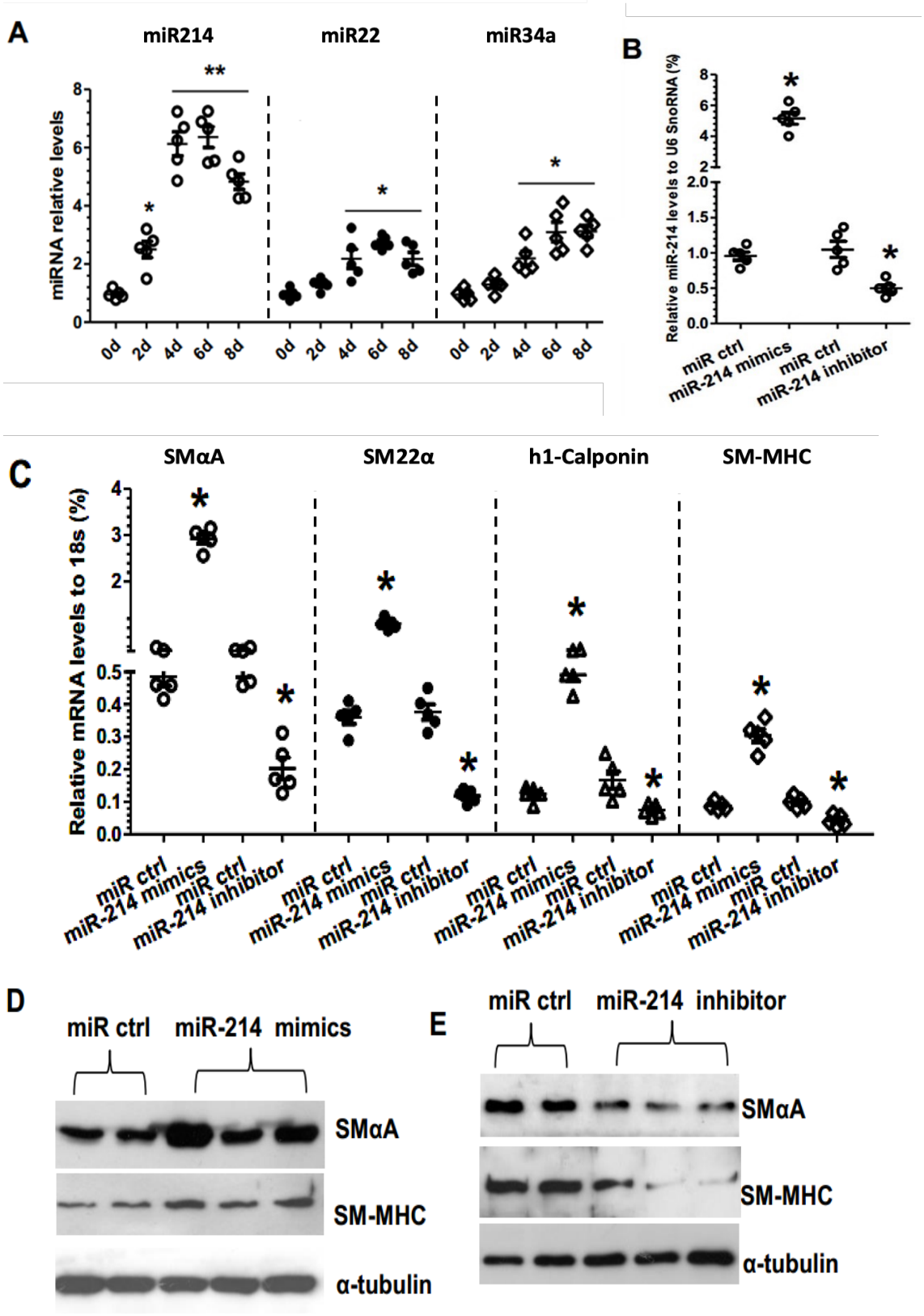


Figure 9. An important role of miR-214 in SMC differentiation from AdSPCs. (A) miR-214 was significantly up-regulated during SMC differentiation. (B-E) miR-214 modulates SMC marker expressions. Day 2 differentiating AdSPCs were transfected with miR-214 mimics, inhibitor or respective negative control (miR ctrl), and cultured for 48~72 hours. Total RNA or proteins were harvested and subjected to RT-qPCR (B & C) and western blot (D & E) analyses, respectively. The data presented here are representative or mean±S.E.M. of five independent experiments (n=5). *P<0.05.

4.2 miR-214 inhibits TNFα induced iSMC differentiation from AdSPCs

ISMC in response to vascular injury are likely to secrete inflammatory cytokines and express cell adhesion molecules (e.g., IL-8, IL-6, and VCAM-1), whereby genes that define the contractile SMC phenotype are suppressed (Orr, et al. 2010). To mimic iSMCs differentiation/generation from AdPSCs *in vitro*, differentiating AdSPCs were incubated with pro-inflammatory cytokine TNFα. As expected, SMC differentiation was succeed by TGFβ1 as evidenced by induction of two SMC-specific genes (SMαA and SM-MHC), while no significant difference was observed with inflammatory genes (IFNγ, IL-6, and MCP-1/CCL2) and S100A4, a reported marker for the rhomboid SMC (R-SMC) phenotype (Brisset, et al. 2007). Interestingly, addition TNFα add into the differentiating AdSPCs primed the differentiated SMC into an iSMC phenotype, with decreased expression levels of SMC genes, but increased levels of the inflammatory genes and S100A4 (Figure 10). Importantly, an increased expression level of miR-214 was observed during the cells were induced by SMC differentiation medium, while such induction was blunted once additional TNFα was added, suggesting a role for miR-214 in these processes.

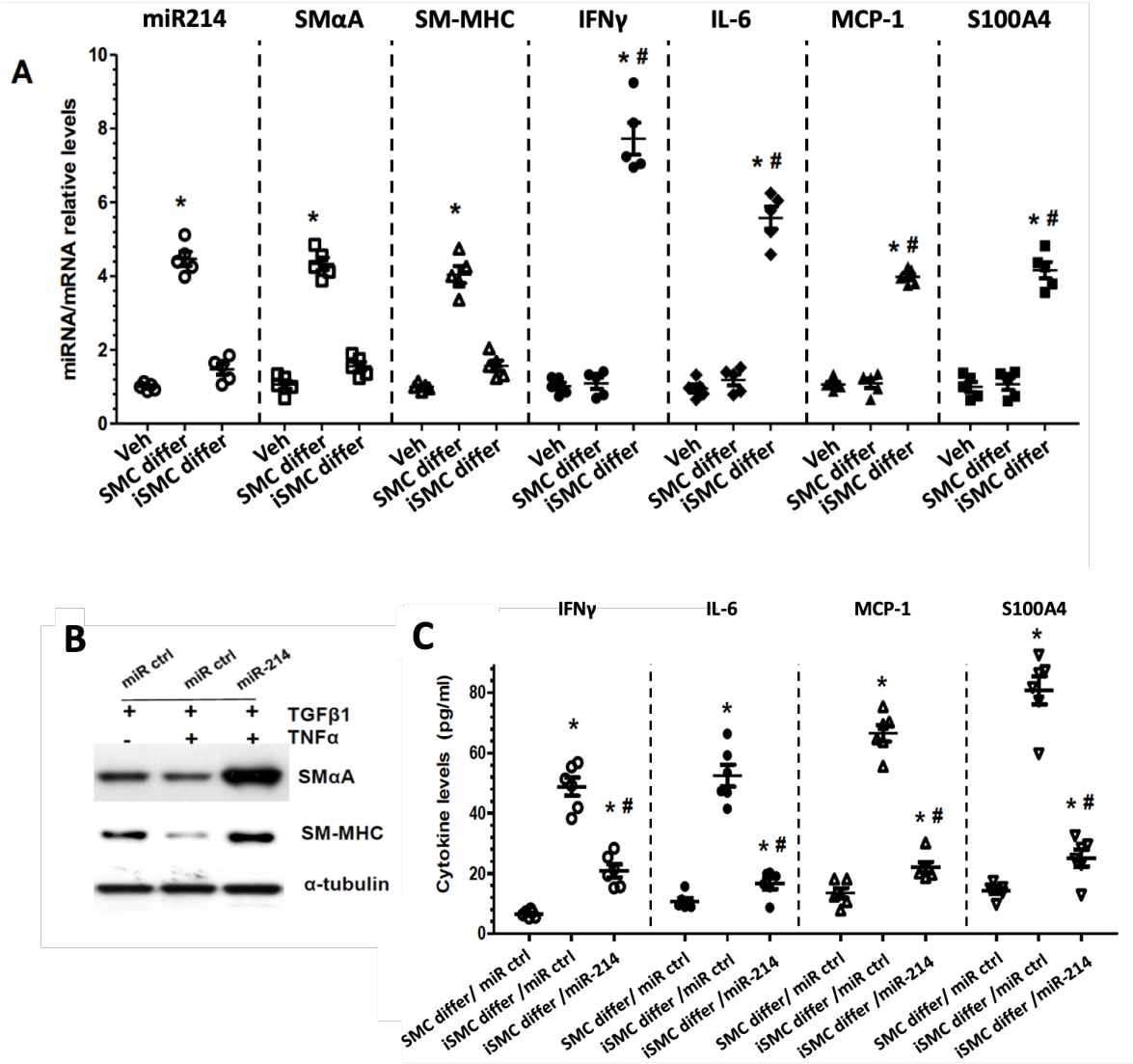


Figure 10. TNFα inhibit the miR-214 expression while induced inflammatory SMC differentiation from AdSPCs. (A-C) AdSPCs were cultured in AdSPC culture medium (Vehicle, Veh), SMC differentiation medium (SMC differ) or iSMC differentiation medium (iSMC differ)for 4 days. Total RNA, protein and conditioned culture medium were harvested and subjected to RT-qPCR (A), Western blot (B), and ELISA analyses (C). The data presented here are representative or mean±S.E.M. of five or six independent experiments (n=5 or 6). *P<0.05 (v.s Veh or v.s SMCs differ/miR ctrl), #P<0.05 (iSMCs differ vs SMCs differ or iSMCs differ/miR-214 vs iSMCs differ/miR-214)

To explore a potential role of miR-214 in iSMC differentiation from AdSPCs, miR-214 mimics was introduced into iSMC differentiation. After 4 days differentiation, miR-214 mimics (miR-214) or negative control (miR ctrl) were transformed respectively and cultured for 48 hours in the same culture medium, total RNA, protein and conditioned culture medium were harvested and subjected to RT-qPCR (Figure 10A), Western blot (Figure 11B) and ELISA analyses (Figure 11C). RT-qPCR data shows that while TNFα incubation significantly inhibited miR-214 expression during the iSMC differentiating but such inhibition was completely reversed by the transfection of miR-214 mimics (Figure 11A). This result also confirmed by Western blot assay (Figure 11B) and ELISA analyses (Figure 11C). Importantly, similar trend with SMC gene expression but an opposite trend for inflammatory genes and S100A4 to miR-214 expression were observed (Figure 11B and 11C). Supporting a notion that miR-214 could rescue inflammatory SMC phenotype induced by inflammation.

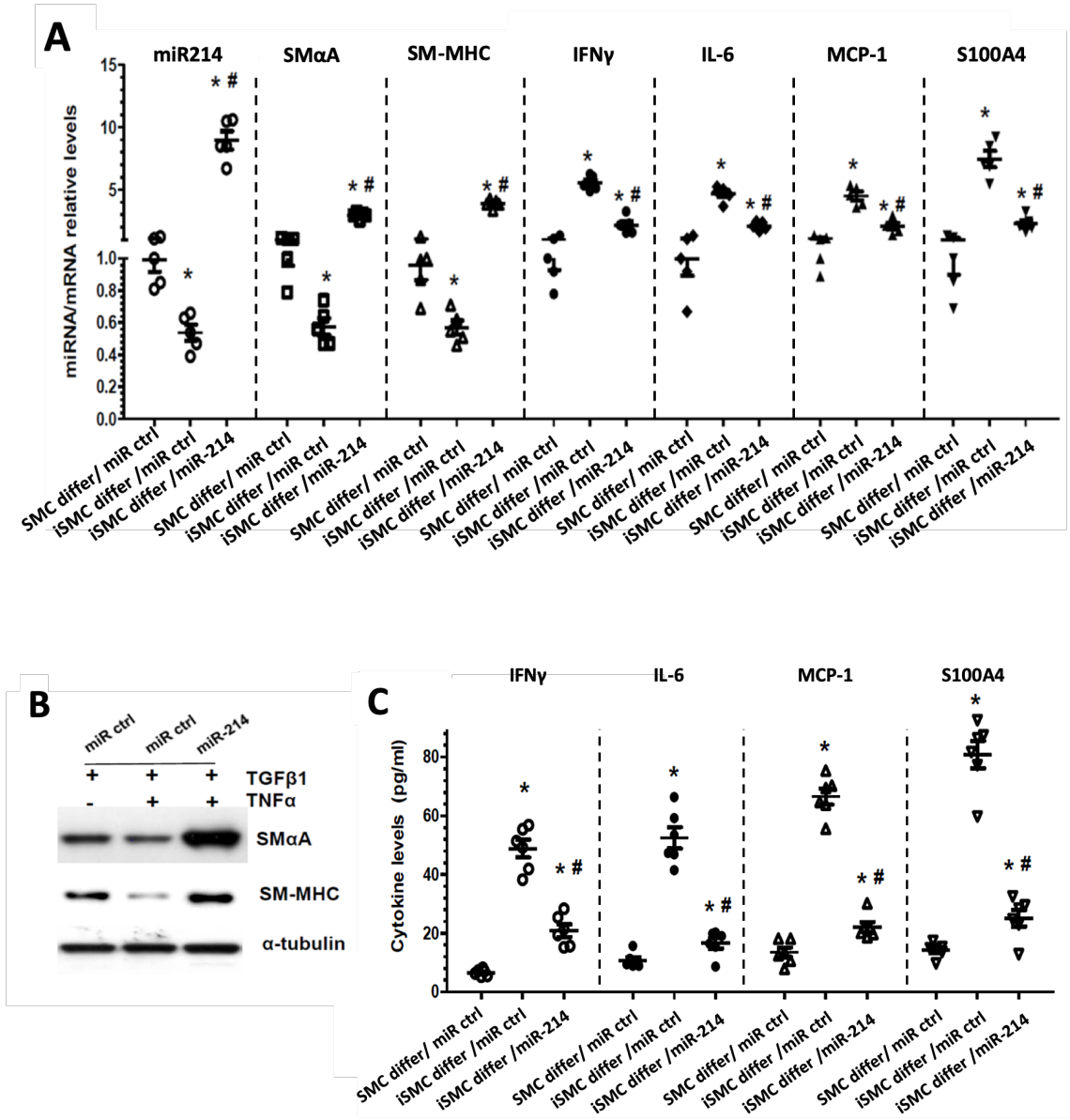


Figure 11. MiR-214 over-express rescued mature/contractile SMC marker expressions, and inhibited TNFα-induced inflammatory cytokine gene expression. AdSPCs were differentiated into SMCs (SMC differ) or iSMC (iSMC differ) for 4 days, then these groups were transfected with miR-214 mimics (miR-214) or negative control (miR ctrl), and cultured for 48 hours in the same culture medium. Total RNA, proteins and conditioned culture medium were harvested and subjected to RT-qPCR (A), Western blot (B), and ELISA (C) analysis, respectively. The data presented here are representative or mean±S.E.M of five or six independent experiments (n=5 or 6). *P<0.05 (v.s SMC differ); #P<0.05 (iSMC differ /miR-214 vs iSMC differ/miR ctrl).

4.3 Shh-GLI1 signal activation and modulation by miR-214 during iSMC differentiation

AdSPCs transfected with GLI reporter constructs were subjected to three different treatments groups: maintained in the AdSPCs culture medium group (Vehicle group, Veh), SMC differentiation group and iSMCs differentiation group. GLI1 reporter activity assays revealed that Shh-GLI1 signalling was significantly activated in TGFβ1-induced SMC differentiation, but its activity was almost abolished in iSMCs (Figure 12A). Additionally, over-expression of miR-214 in iSMCs could reverse GLI1 reporter activity which were reveals another luciferase activity assay experiment (Figure 12B), suggesting that miR-214 could modulate Shh-GLI1 signal pathway during iSMC differentiation from AdSPCs.

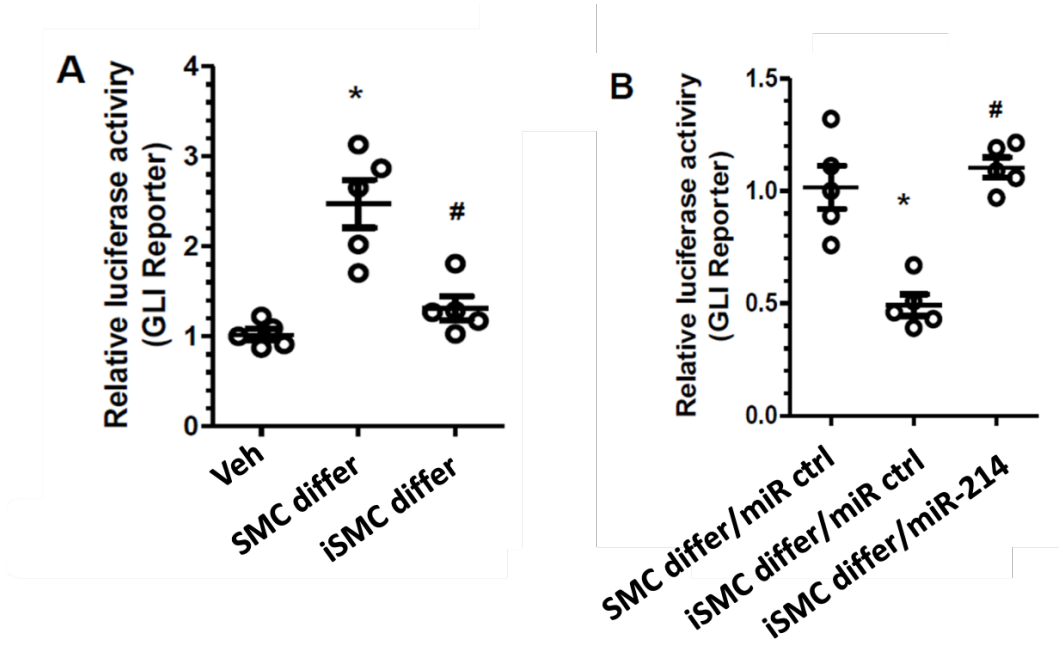


Figure 12. Luciferase activity assays to examine Shh-GLI signalling activity. A; AdSPCs were subject to three different treatment, Vehicle group (Veh), SMC differentiation group (SMC differ) and iSMC differentiation group (iSMCs differ). B; SMC differentiation plus miRNA negative control group (SMCs differ/miR ctrl), iSMCs differentiation plus miRNA negative control group (iSMCs differ/miR ctrl), and iSMCs differentiation plus miR-214 mimic group (iSMCs differ/miR-214). The data presented here are representative or mean±S.E.M. of five or six independent experiments (n=5 or 6). *P<0.05 (v.s veh or v.s SMCs differ/miR ctrl); #P<0.05 (iSMCs differ vs SMCs differ or iSMCs differ/miR-214 vs iSMCs differ/miR-214).

Future more, such a notion was supported by the gene expression profiles, in which data showed that all three main components of the Shh-GLI1 signalling pathway (Shh, PTCH1, and GLI1), as well as its downstream target genes (Wnt1, Wnt4, Wnt9a and WISP1) were significantly activated by TGFβ1 treatment, while such activations were dramatically inhibited by inclusion of TNFα into the differentiating AdSPCs (Figure 13A). Moreover, an opposite phenomenon was observed once miR-214 mimics was introduced into iSMCs (Figure 13B). It is well known that canonical Shh-GLI1 signal activation through releasing GLI1 from cytoplasmic sequestration and translocating into the cell nucleus where it binds to the consensus GLI1-binding element and regulates these gene expression (Zhu and Lo 2010). Western blot assay showed that in iSMCs differentiation model, although increased the miR-214 expression, the total amount of the GLI1 did not change obviously. In contrast the GLI1 protein level in nuclear was significantly modulated and regulated by miR-214 in iSMCs (Figure 13C), which was further confirmed by immunofluorescence staining (Figure 13D). Taken together, these data demonstrated that Shh-GLI1 signalling pathway closely modulated by miR-214 during iSMC differentiation/generation from AdSPCs.

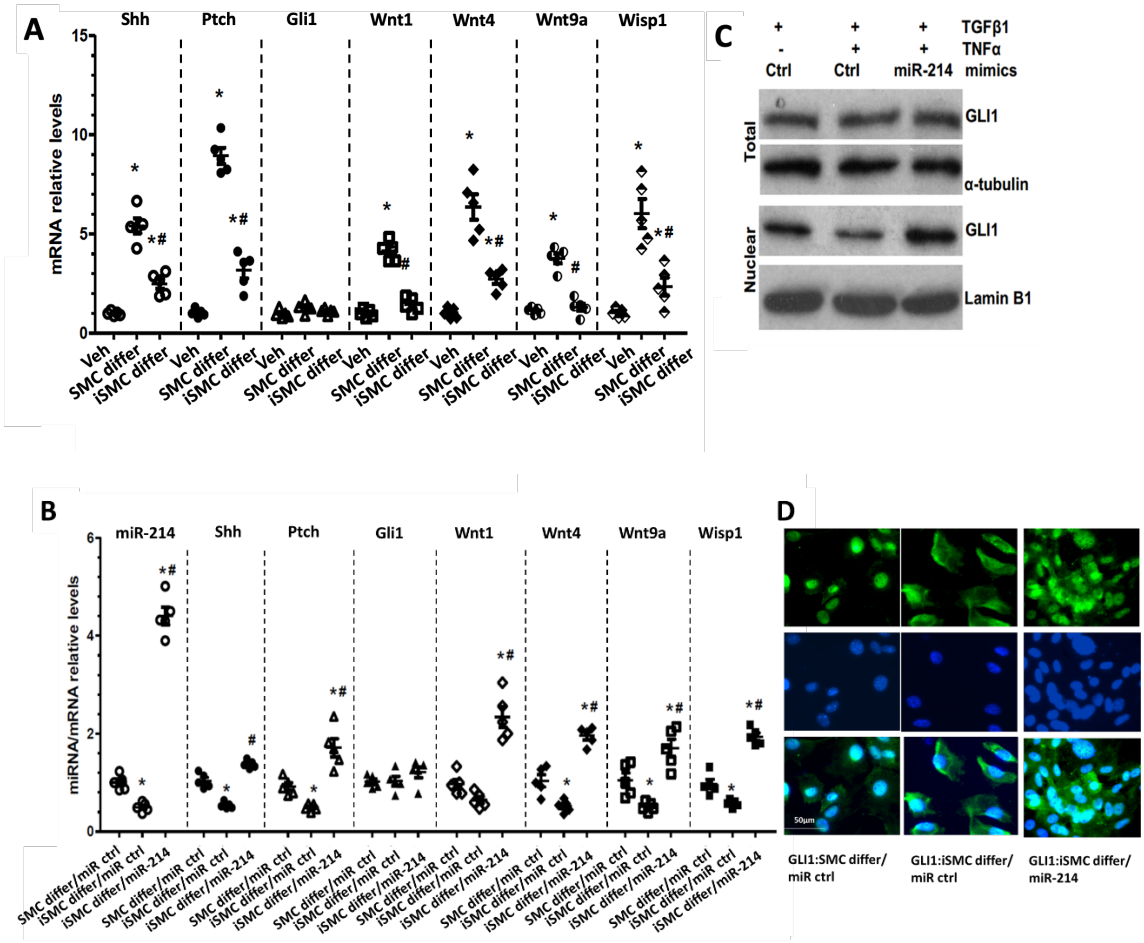


Figure 13. MiR-214 promotes GLI-1 nuclear translocation. (A-B) RT-qPCR detection of Shh-GLI target gene expressio during the SMCs differentiation and iSMCs differentiation. (C-D) GLI1 translocation in AdSPCs during the iSMCs differentiation. Cells were subjected to Western blot analysis (C) immunofluorescence staining against with GLI1 antibody (D). The data presented here are representative or mean±S.E.M. of five independent experiments (n=5). *P<0.05 (v.s Veh or v.s SMCs differ/miR ctrl); #P<0.05 (iSMCs differ vs SMCs differ or iSMCs differ/miR-214 vs iSMCs differ/miR-214)

4.4 Identification of Sufu as the target gene of miR-214 during iSMC differentiation from AdSPCs

Sufu gene expression was detected during the SMC differentiation and iSMC differentiation process. Interestingly, an inverse relationship observed between the expression levels of Sufu (Figure 14A & 14B) and miR-214 (Figure 10A) during SMC and iSMC differentiation from AdSPCs, that Sufu expression Sufu was negatively regulated by miR-214. This rise a question that the relationship between Sufu and miR-214 during the iSMCs differentiation, as demonstrated in miR-214 over-expression (Figure 14C & 14D) and inhibition (Figure 14E & 14F) experiments, respectively. Moreover, Sufu has been reported as a target of miR-214 in zebrafish(Flynt et al. 2007), and both mouse and human Sufu 3'UTR harbours miR-214 binding site(s), indicating that Sufu is the mRNA target of miR-214 during iSMC differentiation from AdSPCs.

To determine miR-214 might directly interact with Sufu gene promoter site and therefore regulate the Sufu express in the cells, the 3'UTR of Sufu containing the conserved miR-214 binding site was cloned into a luciferase reporter. Data from miRNA reporter assay showed that the activity of luciferase from construct harbouring the wild-type Sufu 3'UTR was significantly inhibited by miR-214 over-expression. I found that the conserved miR-214 binding site within Sufu 3'UTR is required for Sufu gene repression induced by miR-214 overexpression in iSMCs, since the inhibitory effect of miR-214 over-expression on Sufu 3'UTR reporter activity was almost abolished once the binding site was mutated (Figure 14G). Taken together, these data clearly demonstrated that Sufu is a true mRNA target of miR-214 and it is negatively regulated by miR-214 during iSMC differentiation/generation from AdSPCs.

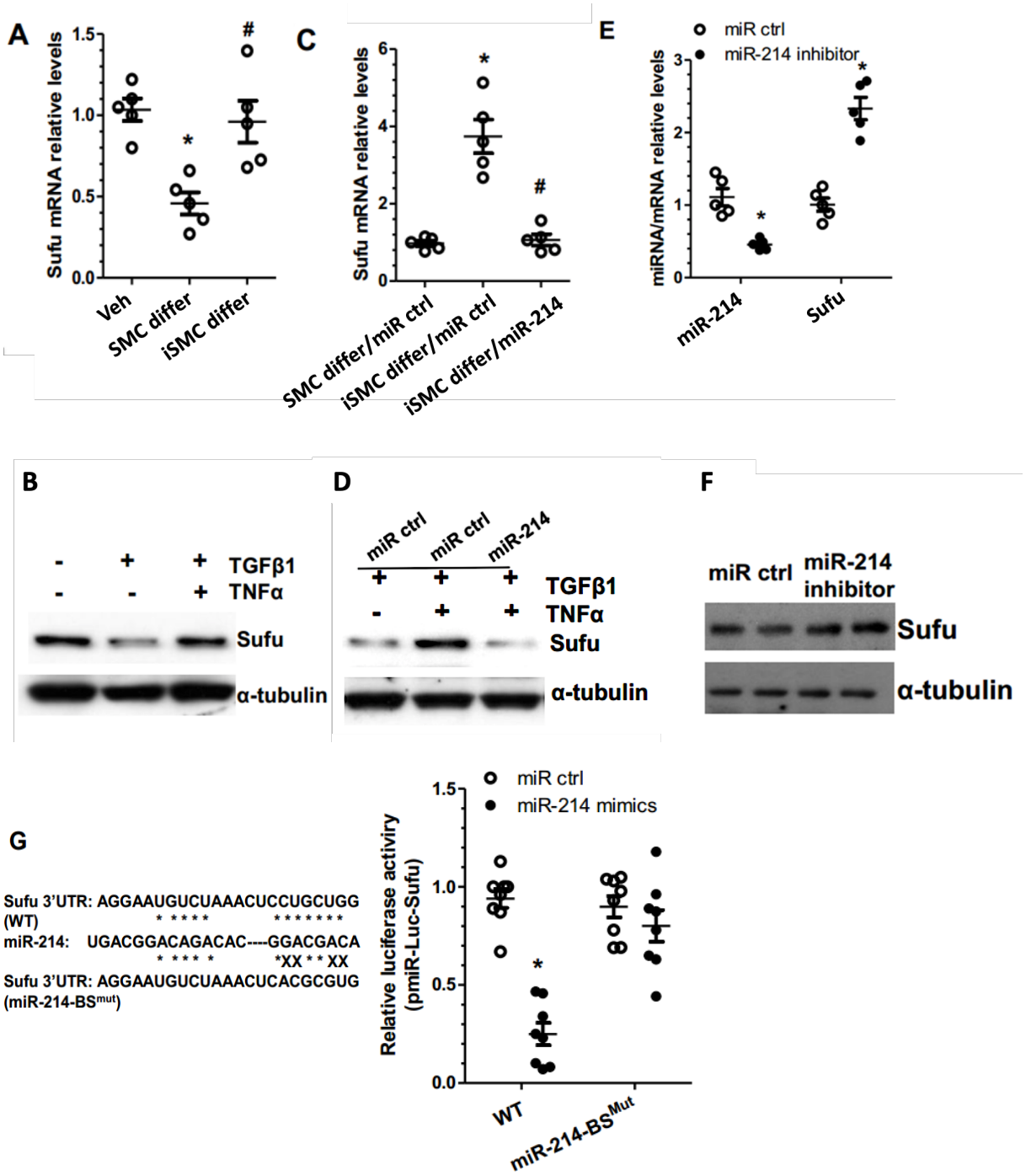


Figure 14. Identify Sufu as the target gene of miR-214 during iSMC differentiation from AdSPCs. (A-B) Sufu is closely modulated during iSMC differentiation from AdSPCs. (C-D) miR-214 over-expression in iSMCs differentiation inhibits Sufu gene expression. (E-F) Up-regulation of Sufu by miR-214 inhibition. Total RNAs and proteins were harvested and subjected to RT-qPCR (A,C,E) and Western blot (B,D,F) analysis, respectively. (G) The binding site is required for miR-214 mediated Sufu gene repression. The potential wild type binding site (WT) of miR-214 within Sufu gene 3'UTR and its mutant (BSmut) are depicted in this illustration (left). miR-214 mimics or negative control (miR ctrl) were co-transfected into iSMCs with wild type Sufu 3'UTR reporter (WT), or miR-214 binding site mutants [miR-214BSmut], respectively. The data presented here are representative or mean±S.E.M. of five (A-F) or eight (G) independent experiments (n=5 or 8). *P<0.05 (v.s Veh or v.s SMCs differ/miR ctrl); #P<0.05 (iSMCs differ vs SMCs differ or iSMCs differ/miR-214 vs iSMCs differ/miR-214).

4.5 Sufu mediates SMC gene expression via modulating GLI1 nuclear translocation

Sufu gene knockdown and over-expression experiments were conducted in the differentiating AdSPCs to study a potential role of Sufu in regulating SMC gene expression. Compared to the respective control cells, Sufu knockdown significantly up-regulated SMC gene expression at both mRNA (Figure 15A) and protein level (Figure 15C). While an opposite effect was observed when over-expressing Sufu in the differentiating AdSPCs (Figure 15B), supporting a notion that Sufu functions as a negative regulator of SMC marker gene expression. Such a notion was further confirmed by SMC gene promoter activity assays (Figure 15D & 15E). Indicating that Sufu regulates SMC gene expression at transcriptional level.

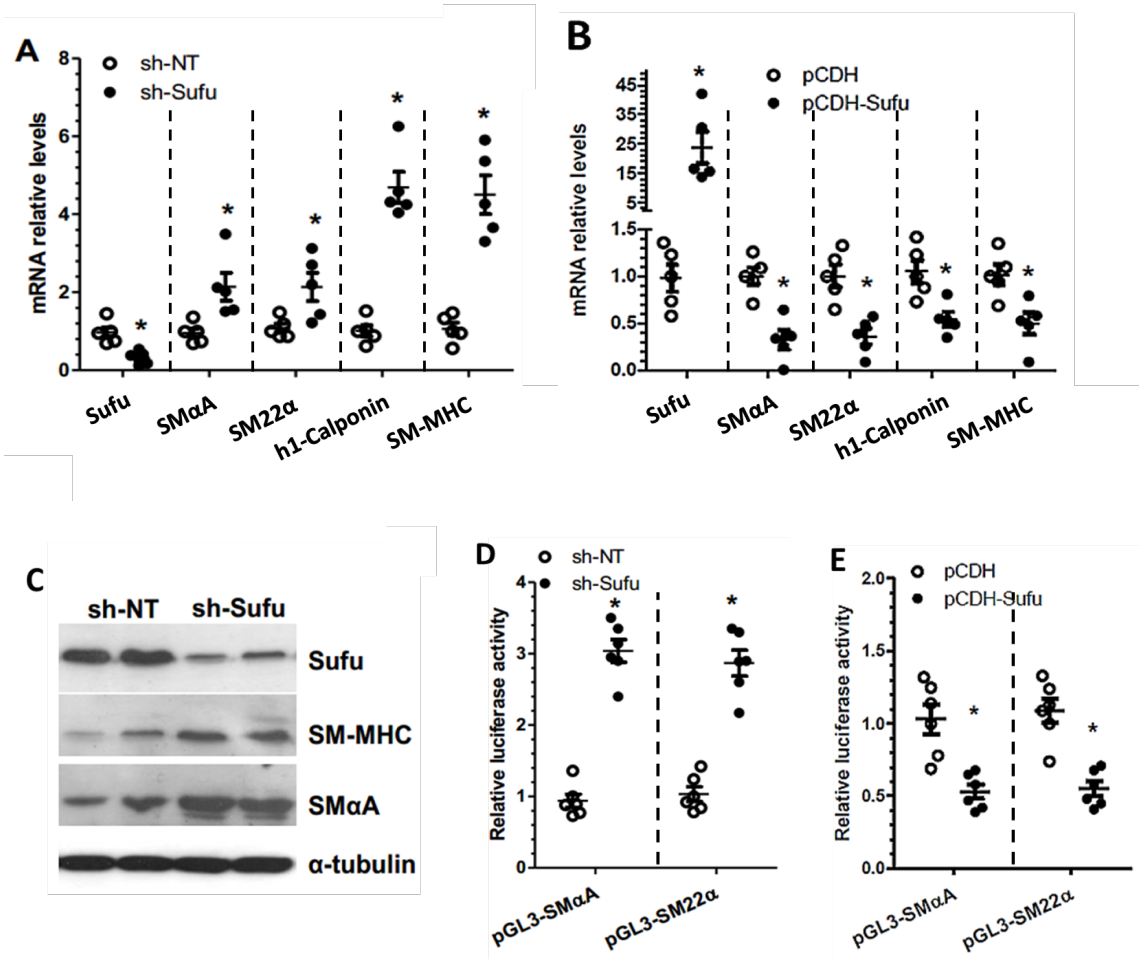


Figure 15. Sufu regulated SMC gene expression in AdSPCs differentiation into SMCs. AdSPCs were infected with non-target (sh-NT) or Sufu (sh-Sufu) shRNA lentivirus as well as control(pCDH-ctrl) or Sufu overexpression(pCDH-Sufu) lentivirus, followed by SMC differentiation. Total RNAs and proteins were harvested and subjected to RT-PCR (A-B) and Western blot (C) analyses, respectively. (D-E) SMC gene promoter activity were regulated by Sufu. AdSPCs infected with sh-NT or sh-Sufu lentivirus as well as pCDH or pCDH-Sufu lentivirus were transfected with SMC gene promoters (pGL3-SMαA or SM22α). Cells were treatment with SMCs differentiation medium. Two days later, total cell lysates were harvested and subjected to luciferase activity assays. The data presented here are representative or mean±S.E.M. of five independent experiments (n=5 or 6). *P<0.05, **<0.01 (vs sh-NT or pCDH).

In order to explore the underlying molecular mechanism of SMC gene transcriptional regulation by Sufu, AdSPCs were infected with non-target (sh-NT) or Sufu (sh-Sufu) shRNA lentivirus, followed by SMC differentiation. Whether GLI1 gene expression level is affected by Sufu inhibition was first examine and RT-qPCR data showed no significant difference for GLI1 mRNA expression level between control and Sufu knockdown AdSPCs (Figure 16A). As mentioned previously, Sufu negatively regulates hedgehog signalling through direct binding with GLI1 and retaining GLI1 in the cytoplasm, thus the cellular location of GLI1 is regulated by Sufu was detected. Data from both Western blot and Immunofluorescence staining shows, there was no big difference between Sufu knockdown and NT control group when comparing the total GLI1 protein, but GLI1 expression were significant increase in the nuclear in Sufu knockdown group comparing with the NT control group (Figure 16B &16C). These assays revealed that Sufu inhibition in the differentiating AdSPCs resulted in nuclear accumulation of GLI1 protein.

Interestingly, I found two possible GLI1 binding sites within SMαA gene promoter. To study the functional importance of these two GLI1 binding sites, GLI1 single (GLI1mut-1, or GLI1mut-2) or combinational (BSmut-1/2) binding site mutant reporters were constructed and used in Luciferase activity assays (Figure 16D). Data from these assays revealed that both GLI1 binding sites are important for Sufu-mediated SMαA gene suppression during iSMC differentiation from AdSPCs (Figure 16E). Finally, chromatin immunoprecipitation (ChIP) assay data revealed that GLI1 directly bound to the regions spanning around GLI1 binding element of SMαA gene promoters, and such binding was dramatically enhanced by Sufu inhibition

(Figure 16F). Altogether, the above data suggest that Sufu represses SMC gene expression by modulating Shh-GLI1 signalling pathway.

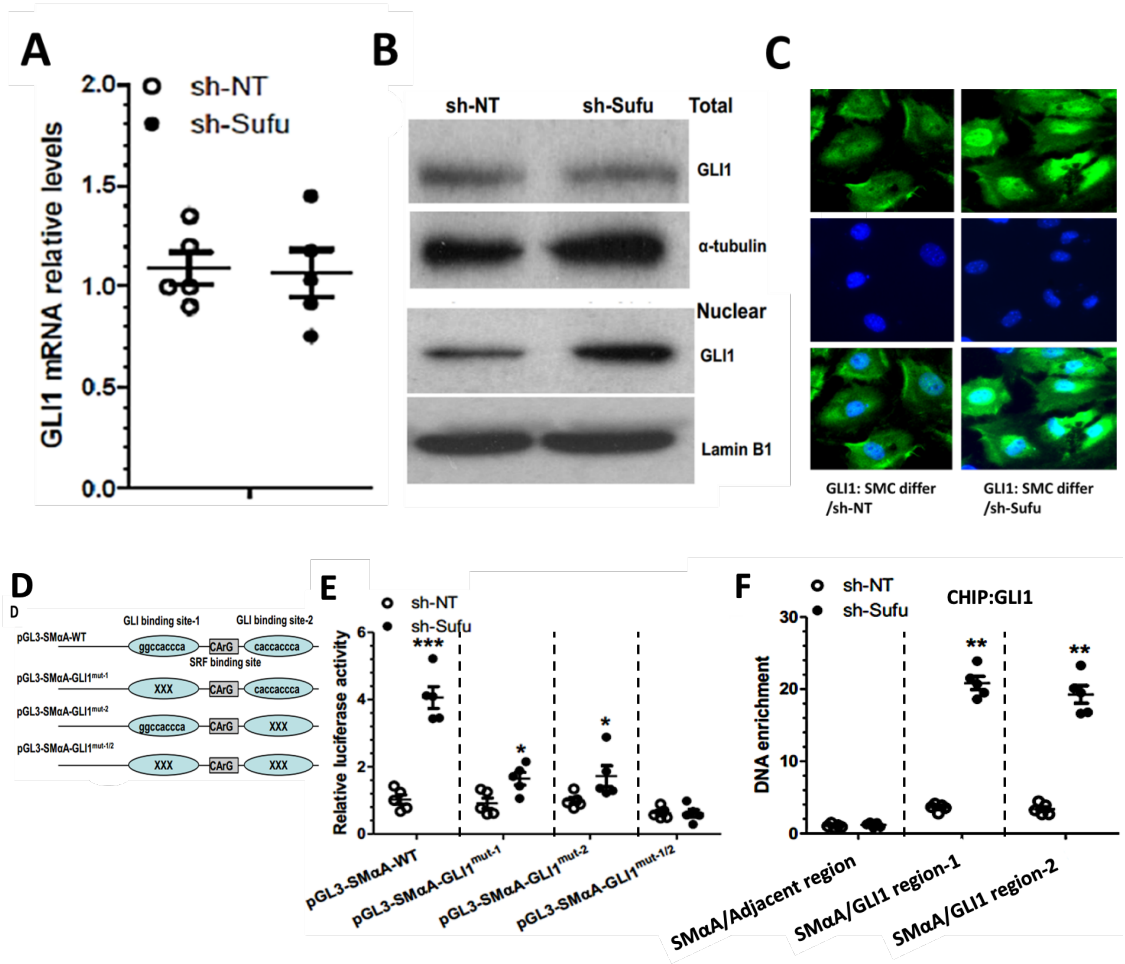


Figure 16. Sufu represses SMC gene expression by modulating GLI1 localisation. (A) GLI1 gene expression was not affected by Sufu inhibition. (B-C) Sufu knockdown promotes GLI1 protein accumulation in nuclei. (D) The potential wild type binding site (WT) of GLI1 within SMαA gene promoter region and its mutants (GLI1 mut). (E) Two GLI1 binding sites within SMαA gene promoter region are required for SMαA gene up-regulation by Sufu knockdown in AdSPCs. (F) Sufu inhibition increases GLI1 enrichment within SMαA gene promoter. ChIP assays were performed using antibodies against GLI1 or normal IgG, respectively. The data presented here are representative or mean±S.E.M. of five independent experiments (n=5). *P<0.05, **<0.01 (vs sh-NT).

4.6 Sufu controls SMC gene expression through regulating transcription factor SRF

Luciferase activity assays with pGL3-Luc-SmαA and pGL3-Luc-SM22α showed that Sufu knockdown in the differentiating AdSPCs significantly activated SMαA and SM22α gene promoter activities (Figure 17A & 17B), and its SRF binding element (CArG) mutation in the two reporters almost abolished their transcriptional activity in response to Sufu inhibition (Figure 17A & 17B), suggesting a role for SRF in Sufu-mediated SMC gene regulation. I found that Sufu over-expression inhibited SRF gene expression (Figure 17C), while Sufu knockdown promoted its expression (Figure 17D). A similar effect was observed in Luciferase activity assays with an SRF gene promoter construct generated in my previous study (Figure17E&F). Moreover, ChIP assays showed that the binding capacity of GLI1 to SRF gene promoters was significantly enhanced by Sufu knockdown (Figure 17G). Taken together, above findings demonstrate that Sufu regulates SMC gene expressions during iSMC differentiation from AdSPCs through modulating the binding of GLI1 to SRF gene promoter and controlling SRF transcriptional activity.

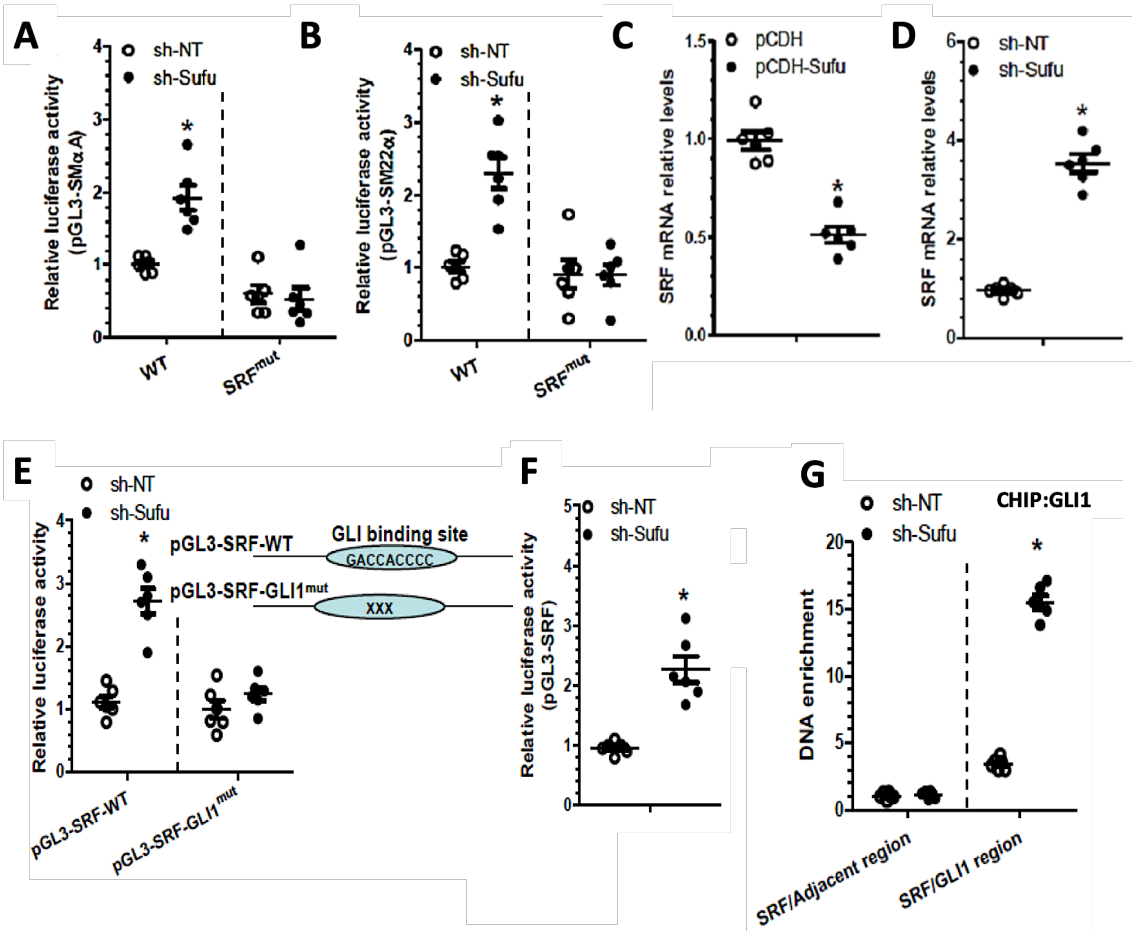


Figure 17. Sufu regulates SMC gene expressions through modulating the binding of GLI1 to SRF gene promoter. (A-B) Serum response factor (SRF) binding sites within SMC gene promoters are required for Sufu-mediated SMC gene repression. (C-D) Sufu over-expression inhibited SRF gene expression, while Sufu knockdown promoted its expression. (E) GLI1 binding site within SRF gene promoter region is necessary for SRF gene up-regulation by Sufu knockdown in AdSPCs. (F) SRF gene promoter activity is increased by Sufu inhibition. (G) Sufu inhibition increases GLI1 enrichment within SRF gene promoter. ChIP assays were performed using antibodies against GLI1 or normal IgG, respectively. Data presented here are Mean±S.E.M of five or six independent experiments (n=5 or 6). *P<0.05(vs sh-NT or pCDH).

4.7 A regulatory role for Sufu-GLI1 signal axis in inflammatory gene expression during iSMC differentiation from AdSPCs

Sufu-GLI1 signal axis has been reported to play a critical role in intestinal inflammatory response, and decreased GLI1 activity predisposes to a heightened myeloid response to inflammatory stimuli in inflammatory bowel diseases (Lees, et al. 2008). I wondered if this axis also plays a role in inflammatory gene expression and iSMC differentiation from AdSPCs. To this end, Sufu specific shRNA lentivirus and over-expression vector were introduced into iSMCs. Total RNAs and conditioned culture medium were harvested and subjected to RT-PCR and ELISA analyses, respectively. RT-qPCR data showed that Sufu inhibition significantly inhibited the expression levels of all three inflammatory genes and S100A4 (Figure 18A), while Sufu over-expression exerted an opposite effect (Figure 18B). A similar effect was confirmed at protein level in ELISA assays (Figure 18C & 18D) as well as at transcriptional level as shown in luciferase activity assays with IL-6 and MCP-1/CCL2 gene promoter reporters (pmIL-6 FL and pMCP-Luc) (Figure 18E & 18F). All these data demonstrate that Sufu serves as a positive regulator in inflammatory gene regulation by negative regulating GLI1 activity during iSMC differentiation from AdSPCs.

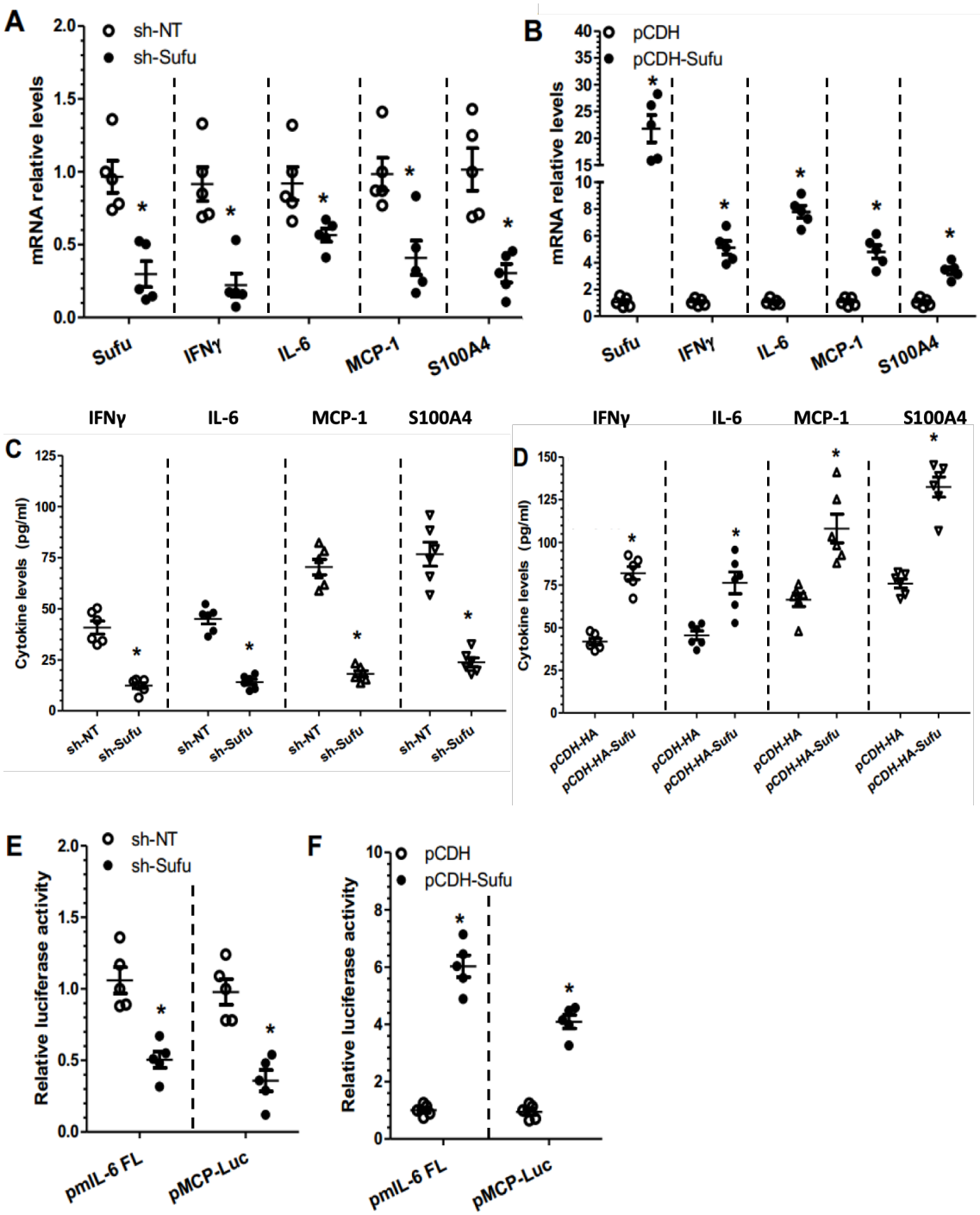


Figure 18. Sufu positively regulates TNFα-induced inflammatory gene expression in AdSPC-derived iSMCs. (A-D) Inflammatory gene expression in iSMCs was positively modulated by Sufu. (E-F) Luciferase activity assays proved that inflammatory gene promoter was positively modulated by Sufu. The data presented here are mean±S.E.M. of five or six independent experiments (n=5 or 6). *P<0.05 (vs sh-NT or pCDH).

4.8 miR-214 over-expression in the injured arteries decreased iSMC formation

To study if miR-214/Sufu axis also play a role in controlling iSMC formation during arterial remodelling, 100µl of 30% pluronic gel containing chemically modified and cholesterol conjugated 2.5nmol miR-214 or Cel-miR-67 agomiRs (negative control) was perivascularly applied to femoral arteries immediately after injury as described in the method. Two weeks later, injured segments of femoral arteries were harvested for H&E staining analyses or cell isolation, respectively. An apparent neointima layer was observed in the injured arteries with control miRNA treatment, while locally enforced expression of miR-214 resulted in a smaller neointima layer (Figure 19A). As expected, cells isolated by enzymatic digestion from normal media layer (SMCs/Sham) constantly exhibited a spindle-shaped phenotype with a classic "hill-and-valley" growth pattern at confluence, while cells isolated from the media/neointima layer of the injured arteries displayed a phenotype resembling R-SMCs as previously reported(Taylor et al. 2002). Interestingly, miR-214 over-expression in the injured arteries reverted the injury-induced R-SMC phenotype to classic one (Figure 19B). Data from immunofluorescence staining revealed cells with R-SMC phenotype were positive for SMαA and IL-6, and miR-214 over-expression significantly reduced iSMC formation (Figure 19C&D). Compared to sham procedure or injured arteries treated with miR-214 agomiR group, expression levels of miR-214 and SMC contractile genes (SMαA and SM-MHC) were significantly decreased in the injured arteries treated with control Cel-miR-67 agomiRs group, while Sufu (miR-214 target gene), IL-6 and S100A4 (iSMC markers) were dramatically increased (Figure 19E). Importantly, perivascular transfection with miR-214 agomiR resulted in an increased level of miR-214 in the injured arteries (Figure 19E), confirming that miR-214 was

successfully over-expressed in the injured arteries. Consequently, the above-mentioned gene expression profiles induced by vascular injury were reversed by miR-214 over-expression. Altogether, above data demonstrate that miR-214 inhibits iSMC formation within the injured arterial walls.

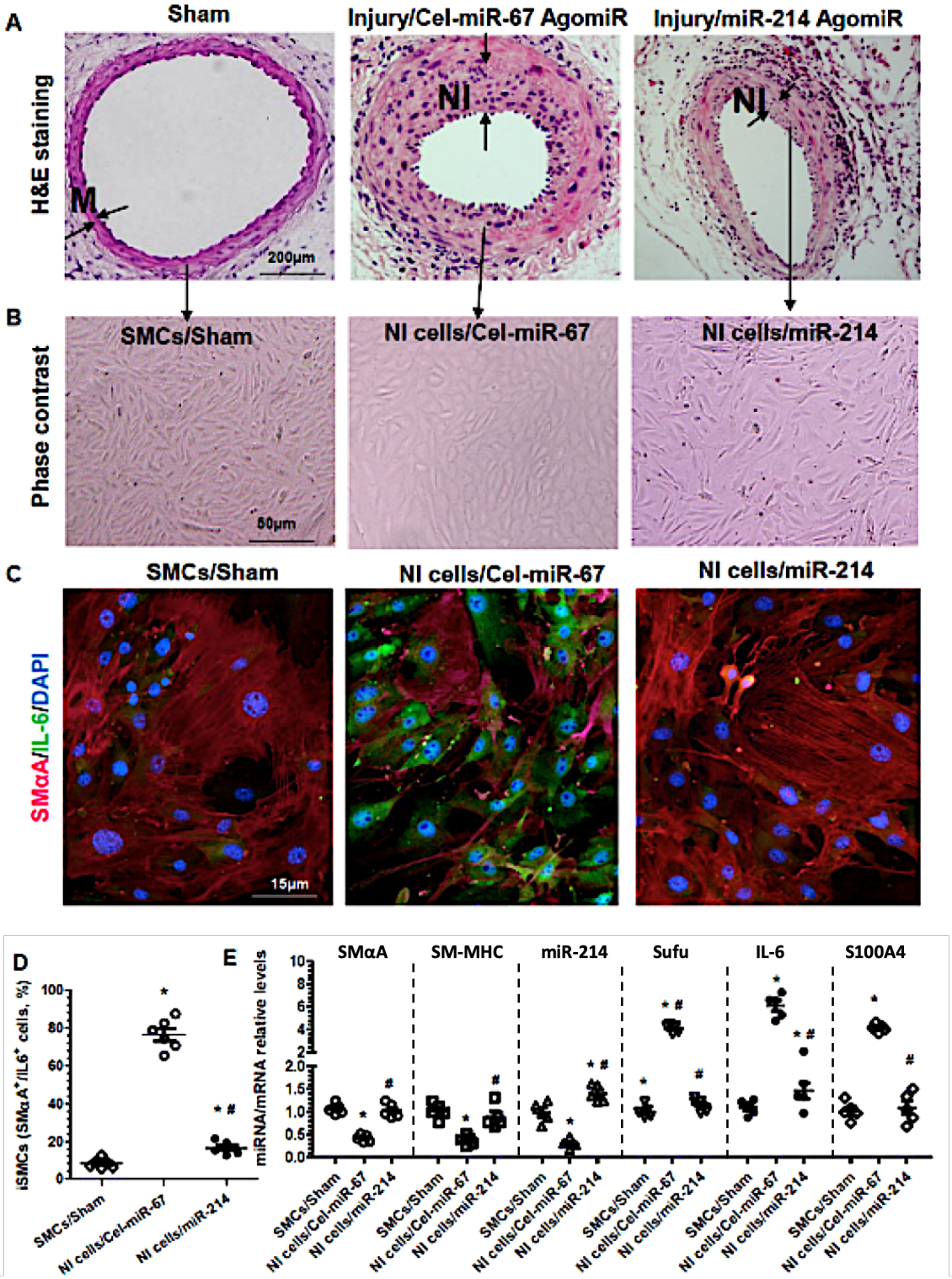


Figure 19. MiR-214 decreases iSMCs during arterial remodelling. Mice underwent a sham arterial injury procedure serve as control (Sham). SMCs and neointima (NI) cells were isolated from media (sham mice) or media/neointima layer and cultured in SMC medium for further experiments, respectively. (B) Phase-contrast images were also taken prior to other analyses and included here. (C & D) Immunofluorescence staining assay with antibodies against SMαA and IL-6. (E) RT-qPCR detection of gene expression. Data presented here are representative images or Mean±S.E.M of five independent experiments (n=5). *P<0.05 (versus sham), #P<0.05 (miR-214 versus Cel-miR-67).

4.9 Transplantation of AdSPCs increases adversely arterial remodeling in response to injury, while miR-214 over-expression in AdSPCs reverses such processes

To investigate a potential contribution of the Ad-SPC derived iSMCs to arterial remodeling, femoral arterial wire injuries were conducted as described in method, AdSPCs infected with Lenti-GFP was immediately applied and packed around injured vessel. The injured segments of femoral arteries were harvested one-week post-transplantation. Triple immunofluorescence staining with antibodies against GFP, SMαA and S100A4 showed that the transplanted AdSPCs could differentiate into iSMCs (cells are positive for GFP, SMαA and S100A4) within the injured arterial walls (Figure 20).

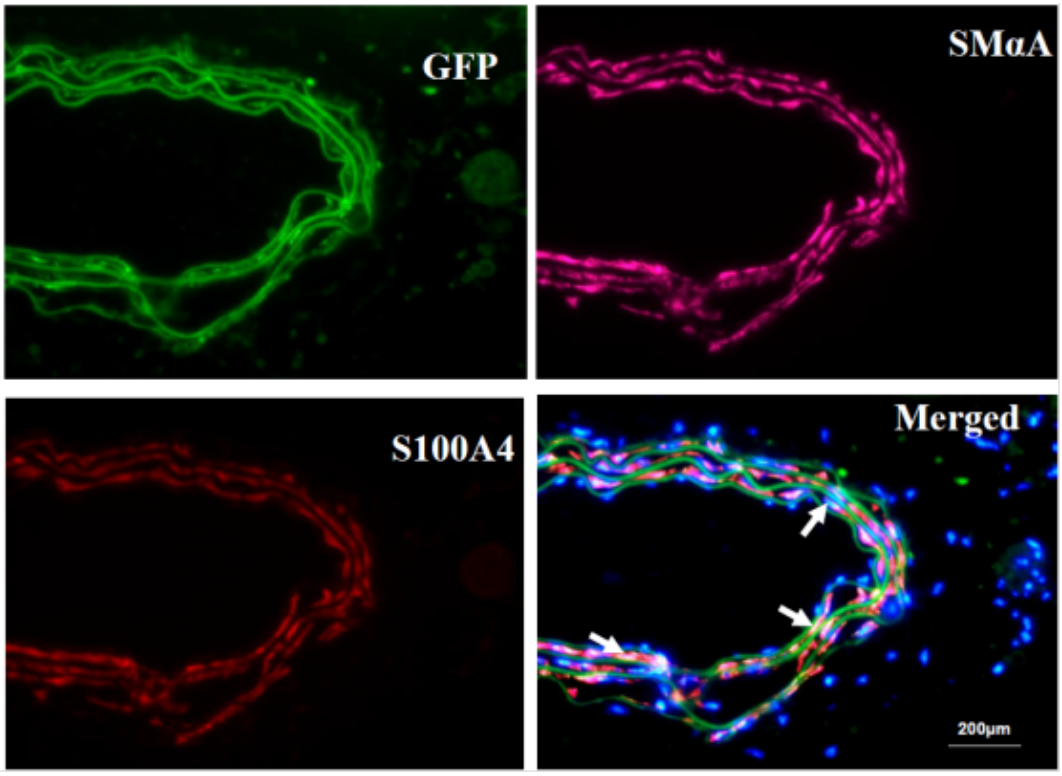


Figure 20. Transplanted AdSPCs differentiate toward iSMCs during arterial remodelling. Injured segments of femoral arteries were harvested after one week and subject to triple immunofluorescence staining with antibodies against GFP, SMαA and S100A4. White arrows within merged image indicate iSMCs derived from transplanted AdSPCs. Data presented here are representative images from three mice.

To further explore the functional implication of miR-214 in controlling iSMC formation and its contribution to vascular remodelling after angioplasty, control vehicle, and AdSPCs infected with Lenti-GFP or Lenti-miR-214 were transplanted onto the injured vessel, respectively. Compared to vehicle control, AdSPC transplantation into the injured arteries resulted in a trend of decrease of miR-214 expression, but significant increased expressions of Sufu, SMαA, S100A4 and IL-6 genes (Figure 21A), further supporting previous observation that the transplanted AdSPCs can acquire an iSMC phenotype during injury-induced arterial remodelling. Importantly, locally transplanting AdSPCs infected with Lenti-miR-214 dramatically increased vascular miR-214 and SMαA levels, but significantly decreased Sufu, S100A4 and IL-6 expression (Figure 21A). Expectedly, a thick neointima was induced by wire injury of the femoral artery after 28 days in the mice treated with vehicle control, and a much bigger neointimal thickening was observed in the mice received the transplantation of AdSPCs infected with control virus which almost two times thicker than the control group (Figure 21B & 21C). Taken together, this data confirmed an inhibitory role for miR-214 in iSMC differentiation/generation from AdSPCs during injury-induced neointima hyperplasia.

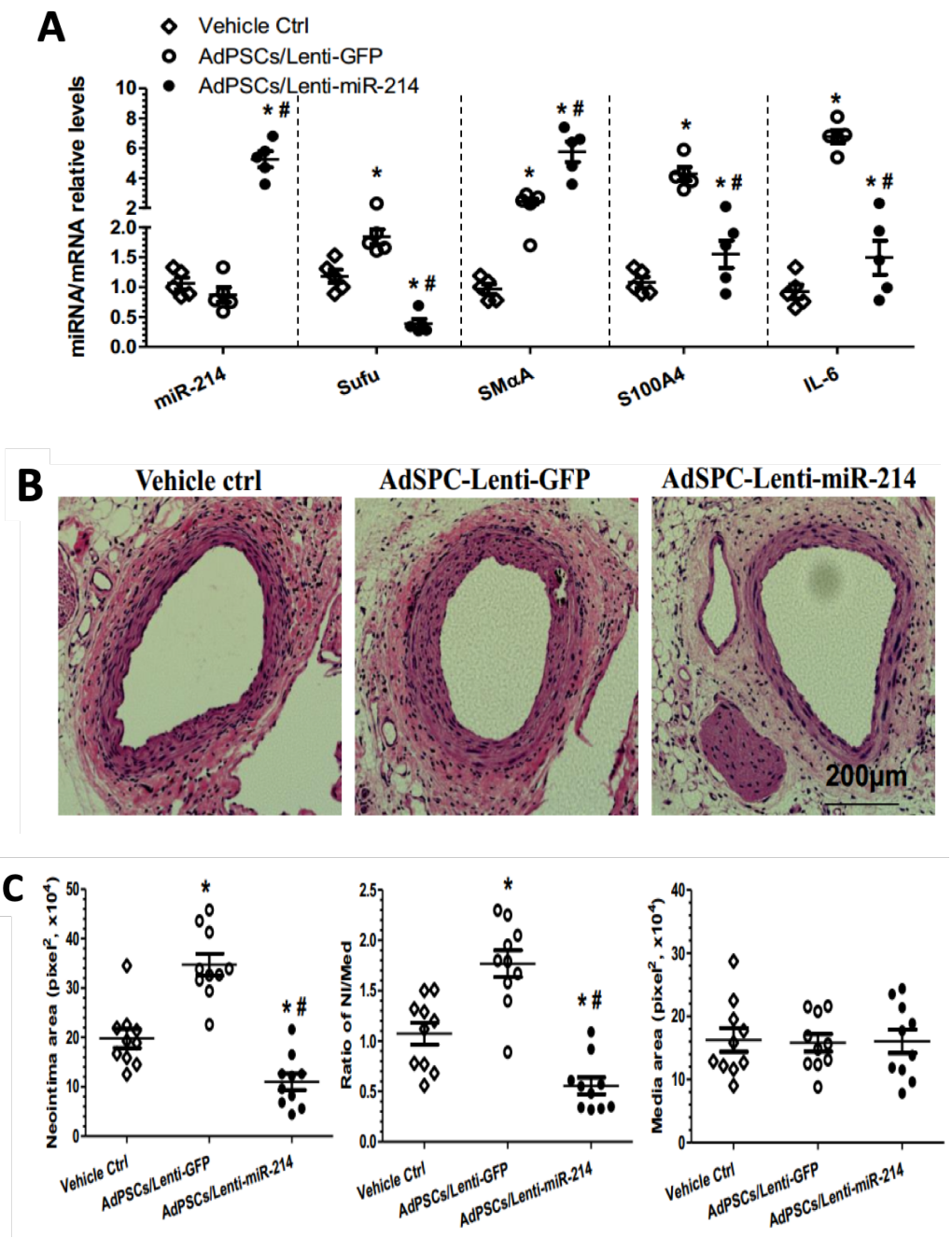


Figure 21. MiR-214 over-expression in AdSPCs reverses AdSPCs increases adversely arterial remodeling function in response to artery injury. (A) RT-qPCR detection of gene expressions in the injured arteries. The data presented in (A) are mean±S.E.M. of five independent experiments (femoral arteries from 3 to 5 mice were pooled for each experiment, n=5 experiments). (B & C) Morphometric analysis of the wire injury induced neointima formation. Paraffin sections from the indicated groups (n=10 mice for each group) were prepared and subjected to H&E staining analyses. Representative images (B) and morphological characteristics including media area, neointimal area, neointimal/media (N/M) ratio, and lumen area (C) were presented here. *P<0.05 (versus vehicle ctrl), #P<0.05 (Lenti-miR-214 versus Lenti-GFP).

4.10 Expression profiles of miR-214 and Sufu in the healthy and diseased human vessels

To further explore the functional relevance of the miR-214/Sufu regulatory axis in a clinical setting, the gene expression levels of miR-214 and Sufu, and their relationship was examined in healthy and diseased femoral arterial specimens. Healthy femoral artery (FA) specimens (n=15) from patients without peripheral arterial diseases and diseased FA specimens (n=15) from patients with peripheral arterial diseases were collected and subjected to RT-qPCR analyses. RT-qPCR experiments were subjected to test gene expression levels of miR-214 and Sufu. Result showed about four to five times decreased expression level of the miR-214 gene, with an increased gene expression level of Sufu in the diseased femoral arteries, compared with healthy arteries (Figure 22A). Importantly, a significant inverse relationship between miR-214 and Sufu in both healthy and diseased femoral arterial specimens were observed in this thesis (Figure 22B). Thus, above human specimens' data provides critical information about the functional relevance of miR-214 and its target gene Sufu in human angiographic restenosis.

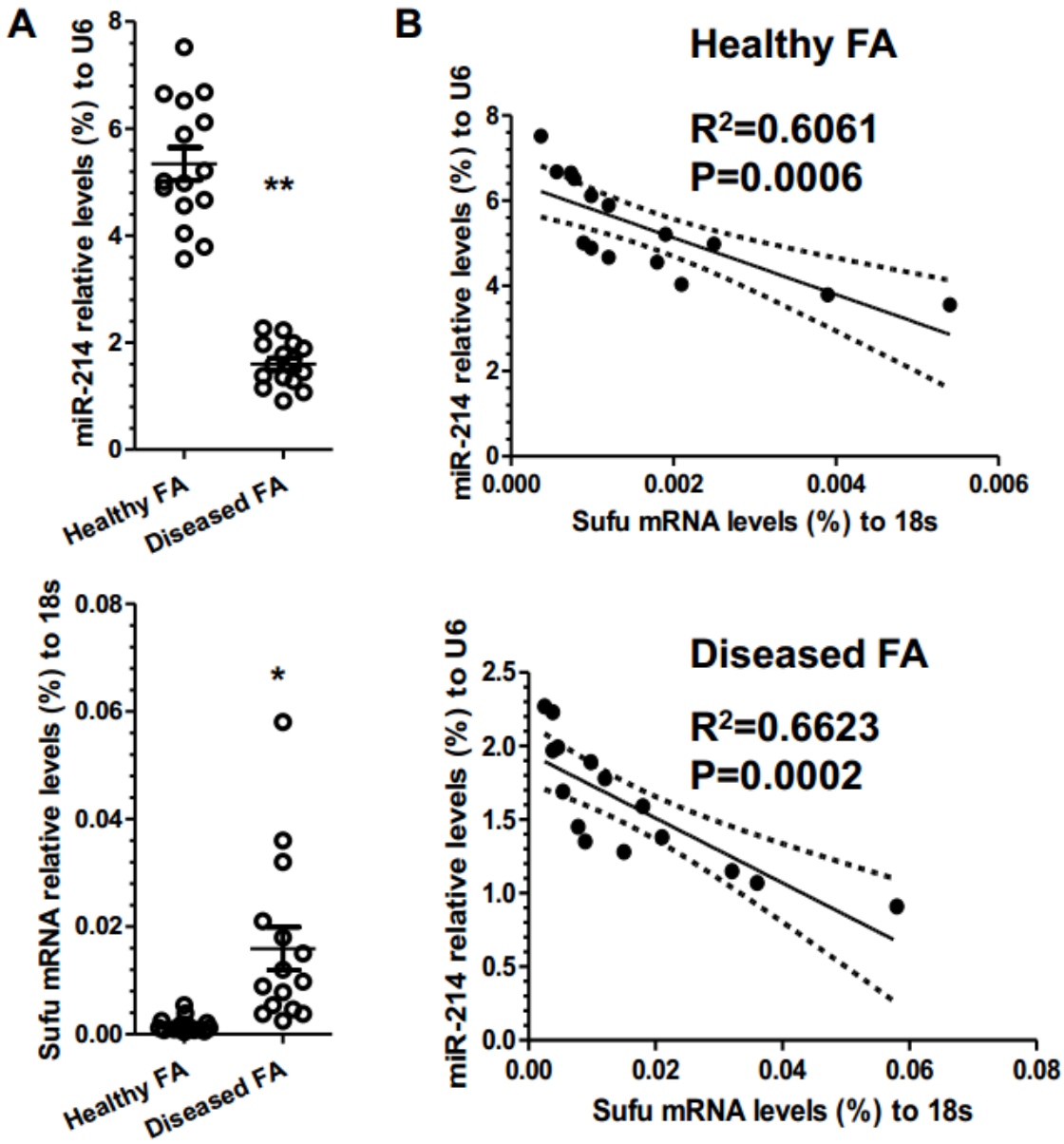


Figure 22. Expression profiles of miR-214 and Sufu in the healthy and diseased human arteries. (A) Expression levels of miR-214 (relative to U6, %) and its target gene Sufu (relative to 18S, %) are shown for each specimen (dots). Bars represent the mean±S.E.M. in each patient group. *P<0.05 (Mann-Whitney U Test). (B) Spearman's rank correlation analyses were carried out to characterize the relationships between the gene expression levels of miR-214 and Sufu in healthy and diseased FA specimens. The solid line indicates the fitted linear regression line; the dotted line indicates 95% CI level. R is Spearman's correlation coefficient between the expression levels of miR-214 and Sufu. P is the P-value indicating the significant level of correlation.

5. Discussion

SMC accumulation and phenotypic transition are the critical steps for vascular remodelling in response to vascular injury. Traditionally it is thought that the accumulation of SMCs in the neointima results from migration of resident SMCs from the arterial media into the intima where SMCs undergo proliferation. Nevertheless, recently research found that there are a large number of vascular SPCs in the vascular wall, which could take part into the iSMCs formation/accumulation in atheroma as well as the restenotic lesions. An early study reported that SPC infusion could reduce the size of atherosclerotic lesions and promote a switch in the plaque phenotype towards a more stable one by reducing macrophage invasion and increasing SMC and collagen content in the plaques (Zoll, et al. 2008). Similar observation was reported in a later study that transplanted AdSPCs contribute to venin-graft atherosclerosis in apoE-deficient mice (Chen, et al. 2013). A new concept was raised by Li and colleagues (Tang, et al. 2013) that vascular disease is likely a stem cell disease. Such a notion was also supported by works conducted by Grudzinska et al (Grudzinska, et al. 2013). They showed that local inflammation in transplanted vessels exerts an effect on surrounding tissue and vessels that leads to phenotypic modulation of SMCs in adjacent vessels. However, those cells did not migrate to the intima and did not contribute to the intimal hyperplasia. Instead, AdSPCs were found to be a prominent source of host-derived cells in the lesion. Therefore, abovementioned evidence supports a notion that both vascular SPCs and SMCs may play important roles in vascular disease, and their contributions to vessel remodelling are highly dependent on the models used and the extent of vessel injury. In the current study, I attempted to address such unmet challenges by uncovering a novel role for miR-214 in modulating SMC differentiation and iSMC generation from

AdSPCs *in vitro* and *in vivo*. Specifically, I find that miR-214 is one of the most up-regulated miRNAs during SMC differentiation from AdSPCs induced by TGFβ1, and demonstrate that miR-214 plays a critical role in this process. Additionally, addition of TNFα into the differentiating SMCs could prime them into an iSMC phenotype, and importantly miR-214 over-expression reverse this priming process. Moreover, this study has provided clear evidence to suggest that miR-214-Sufu-GLI1 axis is one of key signal pathways that are responsible for iSMC differentiation/generation from AdSPCs. Mechanistically, this study has confirmed that Sufu is the authentic downstream target of miR-214 during iSMC differentiation from AdSPCs, and have identified Sufu as a novel transcriptional repressor of SMC contractile genes but an important transcriptional activator of inflammatory genes, respectively. This *ex vivo* and *in vivo* data has shown for the first time that AdSPCs can be induced to differentiate into iSMCs within injured arteries, and that ectopic expression of miR-214 in AdSPCs that are transplanted into the injured arteries can reverse the process of iSMC differentiation from AdSPCs and prevent post-angioplasty restenosis. Importantly, this research also provides clear evidence to support a potential role for miR-214 and Sufu in human atherosclerosis.

MiRNAs are endogenous, highly conserved, and single short strand non-coding RNAs. They have been reported as important regulators for stem cell differentiation (Shenoy, et al. 2014; Kane, et al. 2014) and are emerging as the novel therapeutics or clinical biomarkers for cardiovascular diseases (Barwari, et al. 2016; Gareri, et al. 2016). Although handful miRNAs have been recently reported to play a role in modulating SMC differentiation from stem cells and controlling multiple processes of vascular diseases (Yang, et al. 2018; Yu, et al. 2015; Cheng, et al. 2009), further extensive

investigations are warranted to fully elucidate the exact role of individual miRNAs in SMC differentiation and their significance in cardiovascular development and arterial remodelling. MiR-214 is a member of the miR-199a-214 cluster encoded by a large noncoding RNA, DNM3os, which is transcribed in the opposite strand of the DNM3 gene (Watanabe, et al. 2008). Inconsistent involvements of miR-214 in cancer progression (Chen, et al. 2014; Penna, et al. 2011), and cardiac cell functions and associated diseases (Aurora, et al. 2012; Chen, et al. 2015)have been well-documented in the literature. The possible reasons for such discrepancies are likely due to the fact that different cell systems and animal models were used among these studies, suggesting that miR-214 plays a divergent role in various diseases and the functional implication of miR-214 is cell context-dependent. A function implication for miR-214 in mature vascular SMC functions and injury-induced arterial remodelling has been reported in previous study which reported that miR-214 control both vascular SMC proliferation and migration, two critical cellular events in vascular neointimal lesion formation, and enforced expression of miR-214 in the injured arteries reduces neointima SMC hyperplasia by modulating vascular SMC proliferation (Afzal, et al. 2016). In this thesis, the aim was to provide new perspectives on the role for miR-214 in injury-induced arterial remodelling. Firstly, this thesis addressed the novel concept that AdSPCs are an additional cellular source for neointima SMCs, whereas in previous studies the main focus has been on the traditional concept of mature vascular SMC phenotypic modulation. Secondly, this thesis first observed that miR-214 is the most up-regulated miRNA during SMC differentiation from AdSPCs, and demonstrated that miR-214 plays an important role in SMCs differentiation from AdSPCs. Moreover, this project has confirmed an additional function for miR-214 that miR-214 could revert iSMC phenotype

into a contractile phenotype *in vitro* and *in vivo* even in the presence of strong inflammatory stimuli. Thirdly, this thesis has been able to delineate a novel underlying mechanism for iSMC differentiation/generation from AdSPCs, I found that Shh-GLI1 signalling pathway was activated and modulated by miR-214 during iSMC differentiation, and the location but not the total protein level of GLI1 was modulated by miR-214 in AdSPC-derived iSMCs. Moreover, this project demonstrates that Sufu is the target gene of miR-214 during iSMC differentiation from AdSPCs, and that Sufu regulates gene expression through controlling the cellular locations of GLI1 protein. Specifically, it demonstrates that Sufu is the true target gene of miR-214 in the context of iSMCs and injury-induced arterial remodelling. Sufu exerts its functions by releasing GLI1, the nuclear transcriptional effector of Shh signal pathway, and allowing GLI1 to translocate into nuclei to regulate relevant target gene expression. Finally, these findings are also able to translate from mice into men by documenting the gene expression profiles of miR-214 and Sufu, as well as their relationships in both healthy and diseased human arteries.

One of the new findings in this thesis is that I have successfully established a reliable cellular model to study iSMC differentiation/generation from AdSPCs. Apart from the classical 'contractile' to 'synthetic' phenotypic modulation, extensive evidence also suggests that vascular SMCs can be primed to another distinct phenotype, that is 'proinflammatory' phenotype or iSMCs, in response to vascular injury or inflammatory stimuli. These iSMCs contribute to vascular disease progression and are a pervasive feature in atherosclerotic lesion formation (Orr, et al. 2010) and arterial remodelling (Herring, et al. 2016). However, the majority of previous studies used mature vascular SMCs as a cellular model to study iSMCs and

relevant signal pathways (Ackers-Johnson, et al. 2015; Liu, et al. 2015; Alexander, et al. 2012). Although the existence and functional importance of stem/progenitor cell-derived SMCs in vascular diseases has been increasingly recognised by vascular biology researchers, there has been a lack of a suitable cellular model to study AdSPC-derived iSMCs and the molecular mechanisms involved. In this thesis, I tested the hypothesis that exposure of the differentiating AdSCPs/SMCs to inflammatory stimuli could induce an iSMC phenotype from AdSPCs. Indeed, I found that inclusion of TNFα into the SMC differentiation medium could guide AdSPC-derived SMCs to adopt an iSMC phenotype, which is associated with decreased expression levels of SMC contractile genes and increased expression levels of inflammatory genes. As expected, iSMCs also secrete inflammatory cytokines IFNγ, IL-6 and MCP-1/CCL2. Interestingly, AdSPC-derived iSMCs also express high level of S100A4, the reported marker for R-SMCs (Brisset, et al. 2007). S100A4 belongs to S100 family of calcium binding proteins and was reported involved in tumour metastasis. S100A4 was found up regulate during the oncogenic transformation(Garrett, et al. 2006), this may explain that S100A4 are increased in the rhomboid SMC. Although the iSMCs used here morphologically resemble R-SMCs (Hao, et al. 2002), it would be difficult to draw any assumption that iSMCs are same as or similar to R-SMCs, or simply representing another distinct SMC phenotype, without being side-by-side comparison in terms of their functionalities, pathological contributions to vascular diseases, and global gene/protein expression profiles.

Another new finding in this thesis is that Sufu is the functional target gene of miR-214 in the context of iSMC differentiation from AdSPCs. Although multiple miR-214 target genes have been reported in various cellular

contexts and diseases, for example; NCKAP1 (Afzal, et al. 2016), N-RAS (Liu et al. 2010), ITCH (Chen, et al. 2015a), TFAP2C (Penna, et al. 2011), FGFR1 (Chen, et al. 2014), Quaking (van Mil, et al. 2012a), and Ncx1 (Aurora, et al. 2012), none of them has been shown to play a role in iSMC differentiation from AdSPCs. However, I have demonstrated that the Sufu is a novel target gene of miR-214 which has a role in regulation of iSMCs differentiation from AdSPCs. The Shh pathway is a pivotal morphogenic driver during embryonic development and a key regulator in determining residential vascular stem cell fate (Mooney, et al. 2015). Several components of Shh-GLI1 signal pathway (e.g., Shh, patched-1, patched-2, and GLI1) has been reported to play critical roles in development of the adventitia and maintenance of the resident vascular stem/progenitor cells in the artery wall (Passman, et al. 2008; Kramann, et al. 2015). Importantly, Sufu is a well-known negative regulator of hedgehog signalling. It serves as a docking protein to retain the nuclear effector protein of Shh signalling, GLI1, in the cytoplasm, preventing it being shuttered into nuclei to trigger the transcriptional program (Dunaeva, et al. 2003; Kogerman, et al. 1999), indicating a role for Sufu in vascular stem/progenitor cell development and differentiation. Despite this possibility, no such evidence reported in the existing literature, and the mechanism through which Sufu (or Shh- GLI1 signal axis) is modulated in the context of SMC differentiation from AdSPCs remains elusive. In the current study, I demonstrated for the first time that Shh- GLI1 signal axis is activated during iSMC differentiation from AdSPCs, Sufu is negatively regulated by miR-214, and that Shh- GLI1 signal axis is closely modulated by miR-214/Sufu in iSMCs. Furthermore, I observed that change the expression of Sufu in AdSPCs indeed changed the activity of SMCs gene promoter. Apart from Sufu that has been identified and validated as a bona-fide miR-214 mRNA target in iSMCs in this thesis, NCKAP1 also reported as

another target gene of miR-214 in a previous study (Afzal, et al. 2016). However, no evidence to support the NCKAP1 is the functional target gene during iSMC differentiation from AdSPCs. Such a discrepancy further supports the notion that any given miRNAs play divergent roles under various physiological and pathological conditions by targeting distinct target gene(s), and/or that the miRNAs regulate their target genes in a cellular context dependent manner. One of novel mechanistic findings in the present study is that this research provided clear evidence to support that Sufu could serve as a negative or positive regulator of SMC and inflammatory gene expression, respectively. I demonstrate that Sufu is an important iSMC differentiation mediator by transcriptional regulation of SMC specific genes (SMαA and SM-myh11), SMC transcriptional factor SRF, and inflammatory genes (IFNγ, IL-6 and MCP-1/CCL2). This data showed that Sufu exerts such a transcriptional regulation through modulating GLI1 nuclear translocation. Data generated from luciferase activity and ChIP assays provide multiple lines of evidence to support such mechanism.

Since the discovery of miRNA there has been considerable research focused on this promising small non-coding RNA. Currently, there are about 20 ongoing miRNA clinical trials involved in different human diseases. Significantly, the first miRNA therapy drug called miraviresen, based on miR-122, is currently in clinical trial for HCV infection treatment (Chakraborty, et al. 2017). Comparing with other disease, there is no report on microRNA-based therapy in cardiovascular disease. Although many preclinical works have proved that miR-214 could be a promising therapeutic for protecting cardiac myocytes (Tang, et al. 2016), reducing cardiac fibrosis (Zhu, et al. 2016) as well as arterial remodelling(van Mil, et al. 2012; Afzal, et al. 2016), little data reveals the function of miR-214 in

clinical research. By collecting the femoral artery specimens from patients, my research not only discovered the significant inverse relationship between miR-214 and Sufu in both healthy and diseased femoral arterial specimens, but also revealed that miR-214 expression levels are obviously decreased in the diseased femoral arteries, compared with healthy arteries. This provides promising evidence for future clinical research on CVD including arterial remodeling. Therefore, findings from this thesis and previous one support an important role for miR-214 in iSMC differentiation/generation from AdSPCs, vascular SMC biology, and arterial remodelling induced by injury.

6. Conclusion

In the current study, I focused on the regulatory role of miR-214 in AdSPCs differentiating into inflammatory smooth muscle cells, atherosclerosis and arterial remodelling. This project successfully uncovered the functional involvements of miR-214 in iSMC differentiation/generation from residential AdSPCs as well as in vascular remodelling after injury. During this research, I first observed that GLI1 transport into the nuclear in miR-214 overexpressing AdSPCs during the iSMCs differentiating. The newly identified target gene Sufu is at least partially responsible for miR-214-mediated iSMC differentiation. Specifically, these data have shown that: 1) Sufu negatively regulates SMC gene promoter activity; 2) GLI1 binding site(s) within SMαA and SRF gene promoters are required for these gene transductions regulated by Sufu; 3) GLI1 nuclear accumulation and its binding to the promoter DNA of SMC-specific genes (SMαA and SRF) was modulated by Sufu; and 4) the promoter activity of inflammatory genes (IL-6 and MCP-1/CCL2) is positively regulated by Sufu in iSMCs. Therefore, the findings presented in this thesis suggest that modulating miR-214-Sufu-GLI1 axis could be a potential therapeutic for various vascular diseases such as atherosclerosis and post-angioplasty restenosis (Figure 23).

In conclusion, this PhD study has successfully uncovered a functional role for miR-214 in iSMC differentiation/generation from residential AdSPCs as well as in vascular remodelling after injury (Figure 23).

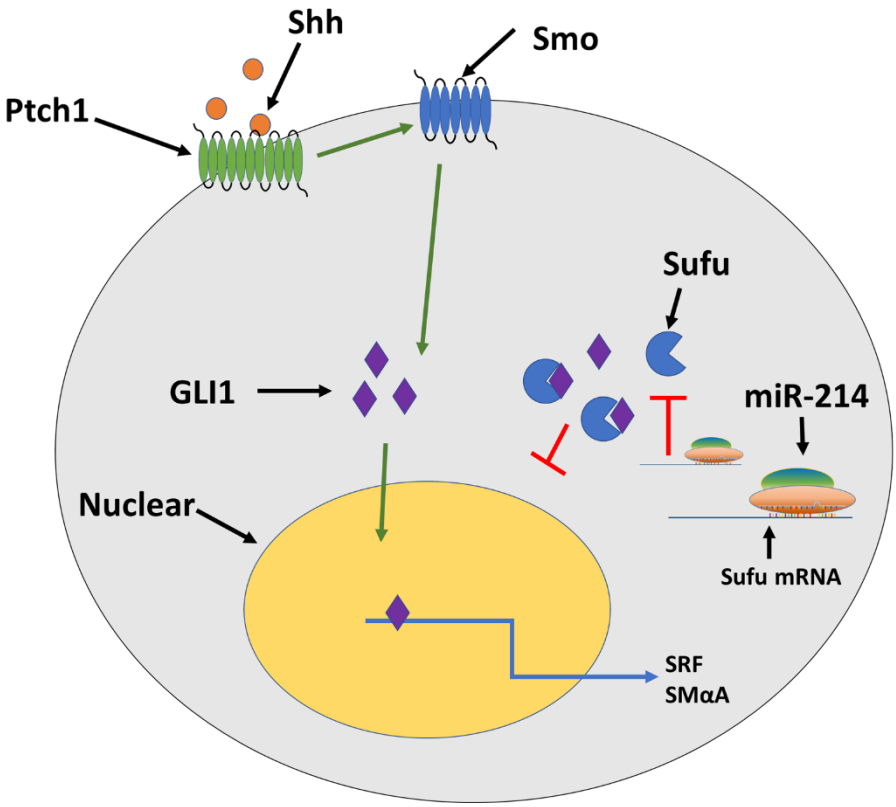


Figure 23. MiR-214-Sufu- GLI1 axis modulate iSMC differentiation from AdSPCs. Sufu block Shh-GLI1 signalling pathway by interact with GLI1 and inhibit GLI1 translocated into the nuclear. MiR-214 complex target on mRNA of Sufu, reduce sufu gene expression could rescue this blocking procedure.

7. Limitations and Future Plan

7.1 Study Limitations

The major limitation of this PhD study would be the fundamental differences between mouse model used in this thesis and the clinical settings of patients with post-angioplasty restenosis or in stent restenosis. Although the vascular injury model used here has been widely used to mimic the pathological processes of neointima SMC hyperplasia and explore underlying molecular mechanisms of arterial remodelling, this model does not completely mimic the conditions prevailing at the micro-environmental level after stent implantation and hence cannot provide us the best way to study the underlying factors at such micro level. Moreover, both atherosclerosis and post-angioplasty neointima formation are mainly incurred at later stages in life, hence the cellular and molecular responses to different inflammatory stimuli are somehow different than in normal young individual. This would represent the 2nd major difference between the animal model (8-week old adult C57BL/6 mice used in this thesis) and the clinical condition (aged human population). Furthermore, hyperlipidemia is considered as a major predisposing factor for atherosclerosis and neointima formation by triggering endothelial dysfunction and accelerating the pathological processes. The normal lipid profiles could be counted as 3rd major difference between the animal model and the patients' actual conditions which normally presents high levels of cholesterol and triglycerides. Finally, although the wire-injury-induced neointima formation is nicely mimicking with post-angioplasty restenosis, but it differs from the in-stent restenosis which occurs after stent implantation.

7.2 Future plan

Despite we have comprehensively demonstrated that miR-214 is critical regulator in iSMC differentiation and potential therapeutic drug target for treating the patients with post-angioplasty restenosis or in stent restenosis, during the course of study we came across some new findings and limitations as discussed in the above section, all of them warrant further investigation.

First, through combined efforts, we have beautifully elaborated the underlying molecular mechanisms of SMC gene regulation mediated by the miR-214/Sufu/GLI1 axis, however the evidence to support such a regulatory role of this axis in inflammatory gene expression is less clear. Further investigation to confirm a regulator role of miR-214 in inflammatory gene expression would expand the therapeutic potential of miR-214 from CVD to other inflammatory diseases, such as asthma, chronic obstructive pulmonary disease, tuberculosis, rheumatoid arthritis, periodontitis, hepatitis, ulcerative colitis and Crohn's disease.

Second, although the main focuses of this PhD study are exploring the functional involvement of miR-214 iSMC differentiation from AdSPCs, the potential importance of this miRNA in other cellular functions including AdSPC migration, proliferation and apoptosis cannot be simply ignored, since all of them have been implicated in injury-induced neointima formation and other CVDs.

Third, as it has been repeatedly mentioned within previous sections that given miRNA could target multiple downstream genes in one or more pathophysiological conditions, it would be important for us to further

examine if there is additional target gene(s) (apart from Sufu) that would also play a role in iSMC differentiation from AdSPCs regulated by miR-214. Fourth, since atherosclerosis is the main underlying cause of ischemic diseases, unravelling a role for miR-214 in atherosclerosis would be paramount to moving this thesis close to clinical application.

Last but most importantly, more rigorous preclinical studies using a more clinically relevant model (such as stent implantation in porcine coronary artery with hyperlipidemia), would be required for further validating the findings presented in this thesis before one could consider its clinical application.

8. Collaborations & Contributions

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9. Publication and Conferences

Journals/publications:

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Conferences:

Oral presentation

1. 27th Jun 2018: William Harvey Research Institute annual Review, London.

Poster presentation

1. 6th-7th September 2018: British Atherosclerosis Society (BAS) Annual Meeting, Cambridge,
2. 4th- 6th June 2018: Joint British Atherosclerosis Society (BAS) &British Cardiovascular Society (BSCR) Congress, Manchester.
3. 15th- 18th April 2019: ESM-EVBO 2019 Conference, Maastricht, The Netherlands.

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